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Comparative Assays on Adrenocorticotrophic Hormone Preparations. (19253)

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It has been established that substances isolated from the pituitary gland, when injected into hypophysectomized rats, will induce an increase in adrenal weight(1) and a depletion of adrenal ascorbic acid(2). These findings led to the development of the adrenal maintenance(3), adrenal repair or weight increase (AWI)(3) and ascorbic acid depletion (AAD)(2) tests for the assay of adrenocorticotrophic hormone (ACTH). It has been reported that ACTH preparations subjected to peptic digestion or acid hydrolysis do not give proportionally equivalent responses in assays by the adrenal maintenance and the ascorbic acid depletion tests(4), and it has been suggested that there are two separate adrenocorticotrophic factors, one which induces an increase in adrenal weight and another which depletes ascorbic acid(5). The present report is concerned with the comparative activity of unhydrolyzed and partially hydrolyzed preparations of ACTH as measured by both the adrenal weight increase and the adrenal ascorbic acid depletion technics.

Methods. ACTH was prepared from swine pituitaries by either acetone-HCL extraction

TABLE I. Comparison of Two Methods of Assay.

Preparation	% hydrolysis	% increase adrenal wt by 5 mg total dose*	Ascorbic acid depletion, mg/100 g adrenal wt	
			2 μg	1 μg
A. ACTH extracted with acid acetone		89.9		
B. Peptic digest of A, #1	43	28.9		
#2	48	17.5		
C. ACTH extracted with glacial acetic acid		92		
D. Peptic digest of C, #1	29	36.7		
#2	46	34.7		
#3	52	6.1		
E. ACTH extr. with glacial acetic acid (Batch 2)		70.6	127	77
F. Same as E, except admin. intrav.		61.4		
G. pH 4.7 precipitate from E after acid hydrolysis		10.5	133	
H. Supernatant from G		11.4	124	
I. Peptic digest of E, #1	46	22.4	200	
#2	51	26.5	135	
#3	70	32.3	154	
#4	75	16.8	142	79
J. Same as I-4, except admin. intrav.	75	20.3		

* Administered intraperitoneally, except F and J administered intravenously.

(6) or glacial acetic acid extraction (7a and b). These preparations were partially hydrolyzed by peptic digestion or by refluxing for several hours in 0.001 N hydrochloric acid. The amount of nitrogen soluble in 5% trichloroacetic acid was employed as a measure of the degree of hydrolysis. The preparations were assayed by the AAD technic of Sayers *et al.*(2), and by the AWI method, which was modified after the description of Sayers *et al.* (8). In the latter method, rats weighing 100 to 120 g each were injected intraperitoneally (except for a few groups which were injected intravenously) three times a day on the 10th, 11th, and 12th days after hypophysectomy. The animals were sacrificed on the 13th day and the adrenals were cleaned and weighed to the nearest 0.1 mg. The AAD technic was modified in that in some cases all of the left adrenals of each group were made into one pool and all of the right adrenals were made into another pool before ascorbic acid determination. All assays were done on groups of 4 to 6 rats.

Results. An acid-acetone extract, designated A in Table I, showed a high degree of activity in the unhydrolyzed state when assayed by the AWI method but two samples of this extract, both hydrolyzed less than 50% by peptic digestion (see B in table), suffered a marked loss of activity when assayed by the same method. Similarly, a glacial acetic acid extract, labelled C in the table, sustained a great loss in activity upon peptic digestion over the range of 29 to 52% hydrolysis as indicated by the AWI method of assay (see D in table). In contrast, it will be seen that when both the AWI and AAD methods are used to assay the same preparation, full activity can still be demonstrated after either acid or peptic digestion when assay is performed by the AAD method, but potency is markedly impaired when the AWI method is employed (see E through I in table). The data in the table (F compared with E, and J compared with I No. 4) show also that similar results are obtained by intravenous and intraperitoneal injections when the AWI method is used.

Discussion. A number of plausible explanations of the results may be presented. There may be two separate and distinct substances possessing adrenocorticotrophic activity(5): one which induces adrenal weight increase and another which depletes adrenal ascorbic acid. If this is correct, then it follows that the adrenal weight factor is significantly less resistant to hydrolysis than the adrenal ascorbic acid depletion factor.

Apparently, difference in absorption rate is not a factor, since similar results with the AWI method were obtained by intravenous and intraperitoneal injection. It is possible that a difference in the inactivation rate of the

preparations in the animal body may be a factor in the results. It is also possible that results may be affected by contamination of the preparations with hormones other than ACTH. Pinto(9) reported that estrogen alone does not increase the weight of the adrenals of the hypophysectomized rat, but when administered along with ACTH induces an increase in adrenal weight greater than that produced by ACTH alone.

Brink *et al.*(10) have reported that ACTH peptides prepared by peptic digestion and tested both clinically in man and by the AAD assay in rats showed activity fully as great as unhydrolyzed ACTH concentrates. Our results, coupled with their observations, suggest that values obtained by the AAD assay will show closer correlation to clinical response induced than will values obtained by the AWI assay.

Summary. In the experiments presented, hydrolysis by peptic digestion or acid-heat treatment induced a marked loss of activity of ACTH as measured by adrenal weight increase, but these treatments had little or no influence upon the activity of the hormone as measured by adrenal ascorbic acid depletion.

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Effect of Cell Concentration on Oxygen Consumption of Leukocytes under Varying Conditions.* (19254)

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Conflicting reports exist in the literature concerning the effect of cell concentration on the metabolism of leukocytes. Barron and Harrop(1) studied the metabolism of human leukocytes in autogenous plasma and found that neither glycolysis nor oxygen consumption increases in proportion to an increase in cell concentration. Soffer and Wintrobe(2) also found that oxygen consumption and leukocyte concentration do not increase proportionally. Ponder and MacLeod(3), however, reported that the oxygen consumption of rabbit polymorphonuclear leukocytes, suspended in buffered saline, is very nearly proportional to the number of cells.

The following work is offered as an attempt to reconcile these two seemingly opposing results and to clarify the extent of the disproportion existing between the cell concentration and the metabolism of leukocytes.

Materials and methods. Exudate leukocytes were collected from normal guinea pigs. Each guinea pig was injected intraperitoneally with 60 ml of a 1% sterile sodium chloride

* This investigation was done in part under a United States Public Health Service fellowship.

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TABLE I. Mean Oxygen Uptake in Serum. Mean respiration rate and standard error of 10 experiments, calculated from first half hour O_2 uptake for each of 5 cell concentrations.

Cell conc., cells/mm³ × 1000	Mean O_2 uptake, 1st ½ hr		Mean respiration rate, mm³ O_2/million cells/ hr	± SE
	mm³	(Range)		
10.5	15	(11– 20)	.96	± .06
21	18	(14– 21)	.59	± .02
42	33	(29– 41)	.52	± .02
84	57	(46– 76)	.46	± .03
168	116	(99–141)	.47	± .02

solution. After 16 hours the peritoneal exudate was withdrawn by gravity with a 16 gauge needle. The cell population at this time interval averaged 70 to 80% polymorphonuclear, and 30 to 20% mononuclear leukocytes. Eight to 10 guinea pigs were used for each experiment at a frequency no greater than once every 7 to 10 days. The pooled peritoneal exudate was centrifuged at 700 rpm for 2 minutes and the supernatant fluid decanted from the sedimented cells. The cells were suspended in 16 ml of a suspension medium by gentle pipetting. From this suspension 4 serial dilutions were made in such a way that the cell concentration of each succeeding dilution was half the cell concentration of the preceding dilution. Cell concentrations varying from 8000 cells/mm³ to 200000 cells/mm³ were used. Three suspension media were used: normal serum, peritoneal fluid, and a physiologic salt solution. Homologous serum was obtained from normal guinea pigs. The bicarbonate ion normally present in the buffer system of serum was reduced(4). The serum was then stored at —20°C until used. Peritoneal fluid was collected, as previously described, and all of the cells were removed by prolonged centrifugation. The bicarbonate ion in the supernatant fluid was reduced and the supernate stored at —20°C. The physiologic salt solution contained the following: NaCl 8.0 g, KCl 0.200 g, $CaCl_2 \cdot H_2O$ 0.147 g, $MgCl_2 \cdot 6H_2O$ 0.203 g, and distilled water to make 1000 ml. Immediately before suspending the cells, 1 part of a 0.5 M sodium phosphate buffer at pH 7.4 was added to 9 parts of the desired suspension medium. In those experiments dealing with the effect of pH, the desired pH of the suspension medium was obtained by varying the pH of the 0.5 M phosphate buffer. In those experiments testing the effect of phosphate, an organic buffer, tris (hydroxymethyl)-aminomethane (5), was substituted for the phosphate buffer. A concentration of 0.05 M tris in serum or peritoneal fluid gave a pH of 7.9.

The oxygen consumption of the leukocytes was measured on 3 ml duplicate samples by the direct method of Warburg. The cells were equilibrated at 37.5°C for 15 minutes and the oxygen consumption measured over a 2-hour period. Oxygen uptake measurements were made in an atmosphere either of air or of 100% oxygen. The reaction vessels were shaken at the rate of 120 per minute. The time from removing the cells from the animals until they reached the bath was 45 to 50 minutes. The pH of an aliquot of each cell suspension was taken at the beginning of the measurement of oxygen consumption and on the contents of each vessel after 2 hours in the bath. The respiration rates are expressed as mm³ oxygen/million cells/hour.

Results. When guinea pig leukocytes respire in buffered serum or buffered peritoneal fluid, the disproportion between cell concentration and oxygen uptake is apparent. In no instance from thirty experiments were the cell concentration and oxygen consumption proportional. The mean oxygen consumption of 10 experiments with the cells suspended in serum is shown in Table I.

Before the cell suspensions reached the constant temperature bath, the pH varied from 7.4 for the lowest cell concentration to 7.1 for the highest cell concentration. After the suspensions had been in the bath 2 hours, the pH varied from 7.4 for the lowest cell concentration to 6.8-6.9 for the highest cell concentration. The difference between any 2 successive cell concentrations seldom was over 0.2 of a pH unit.

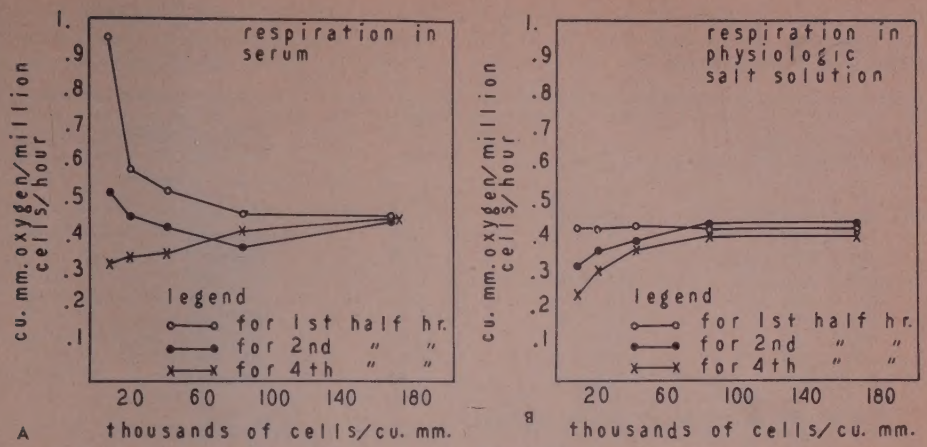

FIG. 1. Relationship of respiration rate of leukocytes to cell concentration at different time intervals. Respiration rates are calculated from the oxygen consumed during the first, the second, and the fourth half hour. Each point on the curves is the mean of 10 values. The curve for the third half hour is consistent with the above.

The effect of the experimental conditions on the disproportion between cell concentration and oxygen consumption was tested. To determine the effect of pH, the individual cell suspensions were buffered to give the lower cell concentrations lower pH values than the highest cell concentration, and so that after 2 hours in the bath the pH range between any 2 cell concentrations was not more than 0.2 of a unit (7.0-7.2). The effect of reducing the ionic strength of the suspension medium was tested at 0.02 M phosphate buffer concentration pH 7.4. The effect of excess phosphate ion was eliminated by buffering the cell suspensions with the tris buffer. To eliminate a possible limited diffusion of oxygen at the high cell concentrations, identical cell suspensions were run simultaneously in an atmosphere of air and 100% oxygen. At least 6 experiments were run at each of the above conditions. In no instance was there a change in the disproportion between oxygen uptake and concentration of the leukocytes.

Fig.1a and b show the difference between the respiration of leukocytes in buffered serum and in buffered physiologic salt solution. The difference between the means of the respiration rates at any 2 cell concentrations, during the indicated time periods, has been statistically evaluated by the t-test. The mean differences in respiration rate for the more critical comparisons are given in Table II. The results indicate that the disproportion between cell concentration and oxygen consumption, when leukocytes respire in buffered serum, is significant. The effect of time on the respiration rate, indicated by the change in shape of the curve at the different time intervals in Fig. 1a, is shown in Fig. 2. The differences in mean respiration rate occurring with time at each cell concentration have been statistically evaluated from data represented by the graphs in Fig. 1a and 2. The standard errors for such differences are of the same magnitude as those in Table II. These calculations indicate that for any one cell concentration, except the highest, the decrease in respiration rate with time is significant. From Fig. 2 it is apparent that the decrease in respiration rate with time becomes less as the cell concentration increases. Fig. 1a shows that with time the respiration rate at each of the 4 lower cell concentrations will pass from a phase wherein each is greater than that at the highest cell concentration, through a phase wherein each is equal to that at the highest cell concentration, to a phase wherein each is less than that at the highest cell concentration. In any one experiment the shape of the curve will depend upon the interval of oxygen consumption from which the respiration rates are calculated and probably upon the time inter-

TABLE II. Mean Difference in Respiration Rate Between Different Cell Concentrations Over 1st Half Hour and 4th Half Hour Oxygen Uptake. Mean difference and standard error calculated from 10 experiments with cells suspended in serum, and from 10 experiments with cells suspended in physiologic salt solution.

Between cell conc., cells/mm³ × 100	Mean difference in respiration rate, mm³ O_2/million cells/hr ± S.E. In serum		In physiologic salt sol.	
	1st ½ hr	4th ½ hr	1st ½ hr	4th ½ hr
105 & 210	.37 ±.05	.03*±.03	.01*±.03	.07*±.04
105 & 420	.44 ±.06	.02*±.03	.00*±.04	.13 ±.04
105 & 840	.50 ±.06	.09 ±.03	.01*±.04	.17 ±.04
105 & 1680	.44 ±.07	.14 ±.03	.01*±.04	.16 ±.03
210 & 420	.07 ±.03	.01*±.02	.01*±.02	.07 ±.03
210 & 840	.13 ±.03	.07 ±.02	.00*±.03	.10 ±.04
210 & 1680	.12 ±.02	.12 ±.02	.02*±.03	.10 ±.03
420 & 840	.06 ±.02	.07 ±.02	.01*±.01	.03*±.02
420 & 1680	.06 ±.02	.12 ±.02	.01*±.01	.03 ±.01
840 & 1680	.01*±.02	.05 ±.01	.02*±.01	.01*±.01

* Difference in respiration rate not significant.

val between removing the cells from the animal until they reach the bath.

During the first half hour, when leukocytes respire in buffered physiologic salt solution, the oxygen consumed is proportional to the cell concentration. This is indicated by the fact that the difference between the means of the respiration rates at any 2 cell concentrations over this time interval is not significant. However, with time the respiration rates of the 3 lowest cell concentrations decrease, so that after the first half hour the disproportion between cell concentration and oxygen consumption is significant.

Discussion. The results indicate that the conflicting reports in the literature are due to observations of leukocyte respiration under different conditions. The observations of oxygen uptake by leukocytes in serum confirm the reports of Barron and Harrop(1), and Soffer and Wintrobe(2). When the oxygen consumption of leukocytes is observed during the first half hour with the cells suspended in a physiologic salt solution, the results tend to agree with the finding of Ponder and MacLeod(3). That a disproportion becomes evident with time is not in agreement with Ponder and MacLeod, who state that the oxygen consumption of their rabbit leukocytes remained uniform over a prolonged period of time. Marinelarena(6), using guinea pig leukocytes which were collected essentially in the same manner as in this study but which were washed repeatedly, found that the oxygen uptake for such cells was uniform over a prolonged period of time. The leukocytes used in this study were not washed, since avoidance of all undue trauma to the cells was desired, as was avoidable delay in transferring the cells from the animals to the bath.

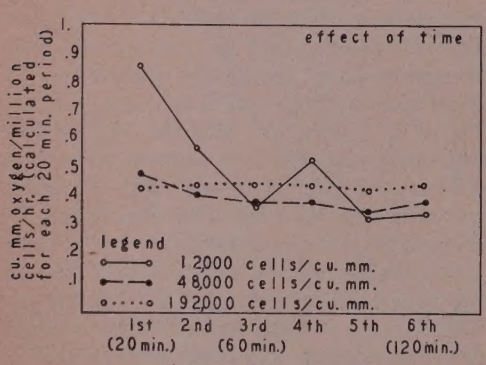

FIG. 2. Effect of time on respiration rates at different leukocyte concentrations with the cells respiring in buffered serum. Respiration rates (mm³ oxygen/million cells/hr) are calculated per 20 min period. Each point on the curve is the average of 6 experiments.

Summary. The conflict between reports in the literature, concerning the disproportion between cell concentration and metabolism of leukocytes, probably is due to observations made under different conditions. Measurements of oxygen uptake of leukocytes suspended in serum show the disproportion between cell concentration and oxygen consumption. When the oxygen consumption of unwashed leukocytes is measured in a physiologic salt solution, cell concentration and

oxygen uptake at first are proportional. However, with time there is a disproportional decrease in the oxygen consumed at the different cell concentrations.

The author expresses his sincere gratitude to Dr. Normand L. Hoerr for the encouragement extended him during the course of this work, and to Dr. George F. Badger for his advice concerning the statistical treatment of this problem.

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Use of Guinea Pig Eye in Study of Intraocular Infections Produced by Mumps Virus.* (19255)

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The injection of mumps virus into the guinea pig eye has been found to produce corneal opacity and result in the appearance of complement-fixing antibodies in the convalescent sera(1). The experiments in this paper deal with the use of the guinea pig eye in (a) the study of the neutralization of mumps virus by specific immune serum and pooled sera from normal mumps susceptible children. Further investigations were performed using the intraocular injection of mumps virus to demonstrate (b) the rise, persistence and decline of complement fixing antibodies in sera, (c) the persistence of mumps virus in ocular tissue, and (d) the corneal reaction and the subsequent appearance of complement fixing antibodies in the convalescent sera after the intraocular injection of 3 recently isolated strains of mumps virus.

Materials and methods. The mumps virus used in experiments I-III was Ender's embryo-adapted strain employed in the earlier study(1); it had undergone 54 serial passages in chick embryos. In the allantoic sac of chick embryos, the virus had a titre (ID_{50}) of $10^{8.3}$ 50% infective doses per ml. In the fourth experiment, 3 recently isolated strains of mumps virus were employed for intraocular inoculation. The JWE_2 virus was recovered from emulsified parotid tissue obtained from a child who had parotitis and meningoencephalitis at the time of his death(2), and had undergone 2 embryo passages. The $WTFE_6$ was recovered from aspirated testicular fluid obtained from a patient with orchitis, and the $135E_8$ from saliva obtained from a patient with parotitis(3). Each of these strains had undergone 6 and 8 embryo passages, respectively. The materials from which these 3 viruses were recovered were injected into the amniotic cavity of 7-day-old chick embryos. The titres of the viruses were not determined. The sera employed in the neutralization tests in guinea pig eyes were from 2 sources. The immune sera were obtained from the authors (V.S.B. and G.R.L.) 18 days after the intramuscular injection of 1 ml of mumps vaccine.† The complement fixing

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† Killed virus from extra-embryonic fluid, V-1061, was kindly supplied by Eli Lilly & Co.

TABLE I. The Use of the Guinea Pig Eye as an Indicator for the Neutralization of Mumps Virus by Immune and Non-Immune Human Sera.*

Material injected	Guinea pig No.	Dilution of virus	No. of reacting corneas	No. of eyes with persisting corneal opacity (days) 1 2 3 4 5 6 7 10 11 12 13 14 15 16	Results of CF tests† Initial sera	Conv. sera
Pooled sera from mumps susceptible children (MSC) + virus	504-506	10^{-3}	4	4 2 0 0 1 1 1 1 1 1 1 1 0 0	<5	<5 <5 <5
	507-509	10^{-2}	1	1 0 0 0 0 0 0 0 0 0 0 0 0 0	<5	40 20 <5
	510-512	10^{-1}	2	2 2 0 0 0 0 0 0 0 0 0 0 0 0	<5	80 40 <5
	513-515	1:2	2	2 1 0 0 0 0 0 0 0 0 0 0 0 0	<5	40 80 40
Virus‡	528-530	10^{-3}	2	2 2 1 0 0 0 0 0 0 0 0 0 0 0	<5	80 80 160
	531-533	10^{-2}	6	6 6 4 4 0 0 0 0 0 0 0 2 2 0 0	<5	160 320 1280
	534-536	10^{-1}	5	4 4 5 5 5 5 5 5 1 1 4 3 2 1	<5	640 320 160
	537-539	1:2	6	4 6 6 6 3 3 4 4 3 3 3 3 3 3	<5	1280 80 160
Immune sera + virus§	516-525	10^{-3} 10^{-2} 10^{-1}	0		<5	<5
	526	1:2			<5	<5
	527	,,			<5	5
Immune sera + normal allantoic fluid	540-542	—	0		<5	<5
Pooled sera from MSC + normal allantoic fluid	543-545	—	0		<5	<5

* Guinea pigs in groups of 3 injected in both eyes with each sample of sera and dilution of virus.

† Normal allantoic fluid controls were negative in each instance of lowest serum dilution tested (1:5). Titres expressed as the denominator of the dilution.

‡ Convalescent serum titres are individually expressed (except No. 540-545) and correspond to the respective guinea pig number.

antibody titre of each of these sera prior to pooling for the neutralization tests was found to be 1:20. Serum was obtained from 3 children who gave a negative history of mumps. The ages of these children were 2.5 to 5 years. Each serum in a final dilution of 1 in 8 was tested against 1000 ID_{50} mumps virus. Each serum-virus mixture was injected into 8 chick embryos. The results showed that the sera prevented the growth of mumps virus in 1/8, 2/8, and 1/8 chick embryos. In each instance the protective titre of serum was less than a dilution of 1 in 8, and the tests were considered negative(4). When the unheated sera were employed in the neutralization tests, the 3 samples were pooled and mixed with the desired dilutions of virus. Each sample of sera was stored in a glass vial at —20°C until used.

Neutralization tests were carried out by injecting mixtures of serum and increasing dilutions of virus into the anterior chamber of the eye. The serum-virus mixtures were incubated for one hour at room temperature prior

to injection. The methods employed for intraocular injection of materials, the use of antibiotics in the inoculum, and the means of determining the levels of complement fixing antibodies in the sera of guinea pigs have been described(1). The normal allantoic fluid employed as a control for eye injection and complement fixation tests was collected from 13-day-old chick embryos. In these experiments convalescent blood was obtained from the guinea pigs on the 16th to 18th day after the injection of virus into the eye.

Experimental. Exp. I. Thirty-six guinea pigs were divided into 12 groups of 3 guinea pigs each. Mumps virus was injected into both eyes of the guinea pigs of 4 of the 12 groups. One group was injected with virus dilutions 10^{-3}, one with 10^{-2}, one with 10^{-1}, and one with 1:2, while the remaining 8 groups were injected with either pooled sera from normal mumps susceptible children or specific immune sera mixed in similar final dilutions of virus. Immune sera and sera from normal mumps susceptible children were each mixed with an equal volume of normal allantoic fluid, and each mixture was injected into both eyes of 3 guinea pigs. The volume of each mixture injected into each eye was 0.03 ml. The results are shown in Table I.

The specific immune serum neutralized the virus and completely prevented the corneal reaction in the 24 guinea pig eyes. Fifteen of the 24 eyes injected with pooled serum from normal mumps susceptible children and virus developed no corneal reaction. The corneal opacity in the 9 reacting eyes was mild and lasted only 24-48 hours. The corneal reaction in the eye of one of the latter animals (No. 506) reoccurred 3 days later.

Of the 24 eyes injected with the various dilutions of virus, 5 failed to develop the corneal reaction. Four of these 5 eyes were injected with virus dilution 10^{-3}. The corneal opacity that developed in 19 of the 24 eyes persisted from 48 hours to 16 days. The corneal reaction in 2 of the 3 guinea pigs injected with virus dilution 10^{-2} persisted for 96 hours and in the third guinea pig (No. 533) for 48 hours. Guinea pig No. 533 developed a second mild corneal reaction and severe iritis 11 days after the initial corneal opacity. Four guinea pigs (534, 535, 536, 539) injected with virus dilution 10^{-1} and 1:2 developed similar recurrent corneal reactions 5 to 11 days after the injection of virus into the eye.

Of the 12 guinea pigs injected with pooled sera from mumps susceptible children, mixed with virus, 5 developed no complement fixing antibodies. The remaining 7 guinea pigs developed relatively low complement fixing antibody titres ranging from 1:20-1:80. Of the 12 guinea pigs injected with specific immune serum and various dilutions of virus, 11 showed no complement fixing antibody in sera diluted 1:5, the lowest dilution tested. The 12th guinea pig (No. 527) injected with virus dilution 1:2 developed a complement fixing antibody titre of 1:5. The 12 guinea pigs injected with the various dilutions of mumps virus without serum developed complement fixing antibody titres that ranged from 1:80-1:1280.

The 6 guinea pigs injected with normal allantoic fluid mixed as a 1:2 dilution with pooled sera from mumps susceptible children or immune sera demonstrated no corneal opacity and no complement fixing antibody in a dilution of 1:5, the lowest dilution tested.

Exp. II. The rise, persistence and decline of complement fixing antibody against the mumps virus was studied in 4 guinea pigs. Each animal was injected in the left eye with 0.05 ml of mumps virus. The right eye was not injected. Sera were collected prior to virus injection and every 4 days thereafter for 28 days. Eight samples of sera were obtained from each animal.

Fig. 1 demonstrates the absence of complement fixing antibody in the sera of the 4

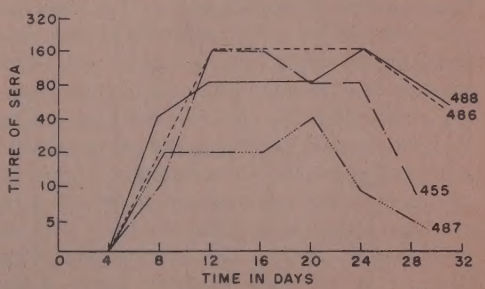

FIG. 1. Rise and decline of complement fixing antibodies against the mumps virus in the convalescent sera of 4 guinea pigs.

TABLE II. Production of Corneal Opacity and Complement Fixing Antibodies in Guinea Pigs as a Result of Intraocular Injection of Recently Isolated Strains of Mumps Virus.

Source of virus*	Guinea pig No.	Eye†	Persistence of the corneal reaction					CF results‡	
			24 hr	48	72	8 days	16 days	Initial sera	Conv. sera
JWE_2	267	R	2+	2+	1+	0	0	<10	160
		L	±	0	0	0	0		
	268	R	4+	4+	4+	4+	4+	<10	40
		L	0	0	0	0	0		
	269	R	±	±	0	0	0	<10	160
		L	±	±	±	0	0		
$WTFE_6$	500	R	4+	4+	3+	3+	2+	< 5	160
		L	0	0	0	±	0		
	501	R	0	0	3+	3+	3+	< 5	80
		L	0	0	±	0	0		
$135E_8$	502	R	±	0	0	0	0	< 5	20
		L	0	0	0	0	0		
	503	R	0	0	0	0	0	< 5	80
		L	±	±	±	0	0		

* Numerical subscript of JWE = number of egg passages.

† Right eye, etc.

‡ Normal allantoic fluid controls were negative in each instance of lowest serum dilution tested (1:5 or 1:10). Titres expressed as denominator of dilution.

guinea pigs before the injection of virus and on the fourth day. On the eighth day significant levels of complement fixing antibodies were demonstrated. These levels persisted until the 20th to 24th day and then commenced to decline.

Exp. III. The next experiment demonstrates the persistence and levels of mumps virus in ocular tissue and the appearance of complement fixing antibodies in the convalescent sera of the guinea pigs after the removal of the virus injected eye. The left eyes of 42 guinea pigs were injected with 0.05 ml of undiluted mumps virus. After virus inoculation, both right and left eyes were removed at intervals of 2 hours to 8 days. There was a total of 15 intervals, the longest being 192 hours and the shortest 2 hours. The tests for virus were performed by injecting ground suspensions of ocular tissue (sclera was discarded) in increasing dilutions into the allantoic cavity of 7-day-old chick embryos. The virus diminished rapidly in the injected eyes, since at 2 and 4 hours the amounts of virus in the ocular tissue showed end points of 10^{-5} and 10^{-3}, respectively. The 10^{-3} level of virus persisted for 2 days. This level of virus then dropped to 10^{-2} and persisted for 2 days. During the next 4 days the end point was 10^{-1} or less. At 8 days, one of 8 eggs showed that ocular tissue still contained mumps virus. The right eyes were tested for virus. In each instance, the test was negative.

Of the 4 guinea pigs that had their eyes removed at 2 hours, 2 developed complement fixing antibody titres of 1:10 while no complement fixing antibody could be demonstrated in the sera of the other 2. In 36 guinea pigs the convalescent sera showed complement fixing antibody titres ranging from 1:10-1:80. These animals had their eyes removed at intervals of 4 hours to 8 days. One guinea pig that had its eyes removed 8 days after virus injection developed a complement fixing antibody titre of 1:320, and one with the eyes removed at 44 hours developed no complement fixing antibody.

Exp. IV. Three recently isolated strains of mumps virus were injected into both eyes of 7 guinea pigs. Control eyes injected with normal allantoic fluid were not included. Two animals were each injected with WTF_6 and $135E_8$ viruses, while 3 were injected with the JWE_2 virus. The volume of undiluted egg virus injected into each eye was 0.05 ml.

As may be seen in Table II, the 3 recently isolated viruses subsequent to 2, 6, and 8 passages in eggs produced an irregular corneal reaction in a majority of the eyes of the guinea pigs. The 7 guinea pigs developed

complement fixing antibody titres that ranged from 1:20-1:160.

Discussion. From the data presented in Table I, it is clear that specific immune serum antibody will neutralize the mumps virus and completely prevent the corneal reaction and the appearance of complement fixing antibodies in the convalescent sera of guinea pigs. When mumps virus is not completely neutralized in serum-virus mixtures, low titres of complement fixing antibodies may occasionally occur in the convalescent sera as was in the case of guinea pig No. 527.

The pooled sera from mumps susceptible children clearly showed an antiviral effect against the mumps virus. The duration of the corneal reaction in those guinea pigs injected with increasing dilutions of virus was 16 days. In contrast, the corneal reaction lasted only 72 hours in those guinea pigs injected with virus mixed with normal sera. All 12 of the guinea pigs injected with increasing dilutions of virus without sera developed complement fixing antibody titres that ranged from 1:80-1:1280. The antiviral effect of the pooled sera from mumps susceptible children appeared to prevent and modify the production of complement fixing antibodies, since 5 of the 12 guinea pigs failed to develop complement fixing antibodies while the remaining 7 developed titres ranging from 1:20-1:80.

The normal pooled human sera employed in the neutralization tests in guinea pig eyes was obtained from children who were considered to be non-immune to mumps virus infections. When serum-virus mixtures were injected into chick embryo, no neutralizing antibody was demonstrated in a sera dilution of 1 in 8. Numerous tests for mumps neutralizing antibody in human sera have shown that the single serum dilution of 1 in 8 seems to distinguish between the immune and non-immune. However, it is possible that the neutralization test in chick embryos is not sufficiently sensitive to demonstrate borderline cases of immunity in children (4).

The results in Table I demonstrate that normal allantoic fluid when mixed with either pooled normal sera or specific human sera and injected into guinea pig eyes plays no part in the corneal reaction or in the appearance of complement fixing antibodies in the convalescent sera.

Within 24 hours after the injection of large amounts of mumps virus into the eye, the corneal reaction occurs. The results in Table I show that mild corneal reactions were observed. The duration of these reactions was 48 hours to 12 days. During the interval of 10 days, they disappeared and reappeared several days later and persisted for variable lengths of time. This recurrent corneal reaction was observed in the eyes of guinea pigs 533, 534, 535, 536, and 539. In the case of guinea pig 533, the corneal reaction persisted for 48 hours and disappeared. Then on the 13th day a severe iritis appeared simultaneously with the second recurrent corneal reaction. The recovery of mumps virus in other experiments 8 days after intraocular injection indicated that the virus was in the ocular tissue at this time. The virus may be in part responsible for the unexplained iritis and the mild recurrent corneal reactions that occurred later in the course of infection.

No complement fixing antibodies were demonstrated in the sera of the guinea pigs before or on the fourth day after the injection of virus into the eye. However, on the 8th day significant amounts of antibody were present. It is possible that neutralizing antibody is in some way related to the final disappearance of virus in the ocular tissue. After the initial precipitous drop in titre, which was a 10,000-fold decrease in virus during the first few hours, the virus continued to diminish slowly in the ocular tissue after the fourth day. In general, the earlier the virus infected eye is removed after virus injection the lower the complement fixing antibody titre in the convalescent sera.

Recently isolated strains of mumps virus when injected into the guinea pig eye produce the corneal reaction and result in the production of complement fixing antibody titres that are comparable to those produced by Ender's strain of virus. The corneal reaction to the 3 viruses appears to be more irregular and less severe than the reaction produced by Ender's virus.

Conclusions. (1) When a mixture of mumps virus with specific immune serum was

injected into the eyes of guinea pigs, the serum completely prevented the corneal reaction and the production of complement fixing antibodies. Pooled sera collected from normal mumps susceptible children, when mixed with mumps virus and injected into the eyes of guinea pigs irregularly prevented the corneal reaction and the production of complement fixing antibodies. (2) Subsequent to the injection of mumps virus into the eye of the guinea pig, complement fixing antibodies appear in the convalescent sera between the 4th and 8th day, persist at significant levels until the 20th to 24th days, and then decline. (3) Mumps virus persists or grows in guinea pig eyes for at least 8 days after inoculation. (4) Chick embryo adapted strains of mumps virus recently recovered from parotid tissue, saliva and testicular fluid when injected into guinea pig eyes produce the corneal reaction, and complement fixing antibodies were demonstrated in the convalescent sera.

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Influence of Temperature upon Radiation Sensitivity of Thermophilic and Mesophilic Bacteria. (19256)

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Thermobiosis has been the subject of considerable speculation, but of little experimentation. Gaughran(1) indicated that there was no fundamental difference in the effect of temperature on the respiratory systems of stenothermophilic and mesophilic bacteria. He postulated that the thermophilic organism may produce a substance to protect its proteins against heat. Allen(2) postulated that growth at thermophilic temperatures was possible because of an increased rate of synthesis of cellular proteins. She pointed out the relative ease with which thermophilic strains may be obtained from mesophilic cultures, and suggested that a very simple genetic change must be involved. Militzer *et al.*(3-5), and Georgi *et al.*(6) have recently shown that a number of thermophilic enzymes are much more heat stable than are those of mesophiles. The influences upon radiation sensitivity of temperature and of oxygen tension during irradiation have been examined by several workers. Hollaender *et al.*(7) reported an increased X-ray sensitivity when *Escherichia coli* was irradiated in an aerobic atmosphere. The same was also observed at low temperatures (2°C), but only when oxygen was present. Wyss(8) reported that hydrogenase-producing organisms can resist radiation much more effectively in an atmosphere of hydrogen than of nitrogen or methane, and that an oxygen atmosphere markedly increased their sensitivity. The demonstration of heat-stable enzymatic proteins in thermophiles suggests the presence of heat-stable nuclear proteins. Since thermophilic strains may be readily selected from mesophilic cultures, it seems doubtful that an independent genic alteration would be necessary for rendering each specific protein heat-stable. It appears more reasonable to believe that the modification resulting in an increased heat stability is established in the non-specific protein molecule before its final specificity is determined, *i.e.*, early in its synthetic sequence.

The present investigation was undertaken

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in an attempt to determine whether the intact genetic apparatus is heat-stable in thermophilic organisms when subjected to ultraviolet radiation at different temperatures should give data indicative of their relative nuclear heat-stability, since this radiation is heavily absorbed by such material. A heat-stable protein would not be expected to be affected by radiation as strongly at different temperatures as would be a heat-labile one. The latter would suffer concomitant heat and radiation denaturation.

Experimental methods. Spore suspensions of *Bacillus stearothermophilus*, strain No. 1503 (obtained from the National Canners Association), and *Bacillus globigii* were prepared from Difco nutrient agar flats (containing 0.2% soluble starch) which had been incubated for 5 days at 55 and 37°C, respectively, (at which time most of the vegetative cells had lysed). The spores were then washed with M/200 phosphate buffer, resuspended in the buffer, and filtered through cotton to remove the clumps. These suspensions were irradiated in the buffer at pH 7.0, either in large petri dishes containing 50 ml of spore suspension, or in special quartz cuvettes (17 ml) designed for irradiation through the side of the upright vessel. The latter suspensions were agitated during irradiation with air or hydrogen gas, which was brought to the proper temperature prior to its being bubbled into the suspension by passing it through a coil immersed in a water bath. Suspension temperatures during irradiation were 4, 24, 37, 55, 75, 90, and 100°C, and suitable viability controls were effected for each series. Temperatures above 24°C were held constant by the use of a small thermostatically controlled hotplate. The buffer was heated to the proper temperature prior to the addition of spores in order to limit possible germination during a slow heating, and the suspension was then immediately irradiated. Predetermined amounts of heated buffer were added at intervals where needed to compensate for loss by evaporation. Water vapor over the open plates was removed by passing a stream of sterile air directly over the surface of the spore suspension. Aliquots were withdrawn at intervals and plated for survivors and zero-point mutants and for inoculation into Difco nutrient broth tubes for the later determination of end-point mutants. Cultures were plated as soon as turbidity appeared in an effort to minimize selection factors, since growth rates of the streptomycin resistant and sensitive strains were found to be the same during the initial stages of growth, whereas the final turbidity attained by the resistant strains was lower. A level of streptomycin (2.0 μg/ml) was selected which yielded 1.1 and 0.5 spontaneous mutants/million respectively, for *B. globigii* and *B. stearothermophilus*. The colonies appearing upon this level of antibiotic were tested further for confirmation of their resistance. The activity of the level of streptomycin employed remained constant over the 3 or 4 days involved in incubation at 55°C. Mutation incidence was also determined in control experiments using the triphenyltetrazolium method of Lederberg(9) for the detection of fermentation mutants, and in all cases the results were found to be comparable with those obtained using streptomycin-resistance as the criterion of mutability. The incidence of thermophilic biochemical mutants(12) was too low to enable comparisons to be made. Determination of zero-point mutation incidence in spore experiments was complicated by germination factors, and such determinations yielded erratic results. Therefore, only end-point mutation incidences were reported. In early experiments the spores were uniformly heat-shocked for 10 minutes at 95°C(10) but such treatment applied during experiments designed to determine an effect of temperature itself is not desirable. Mefferd and Campbell(11) have shown that the same order of germination of thermophilic spores can be secured when a plating medium containing 10^{-6} parts furfural is substituted for a heat shock. Direct plating upon the starch-nutrient agar medium containing furfural was, therefore, employed here. No detectable photoreactivation was noted in the irradiated spores of any strain. Because of the many variables present, very carefully and empirically standardized conditions were employed throughout all operations. Even so, results of individual experiments varied to a small degree, but trends were constant.

Experimental results. Survivor and endpoint mutation incidence curves for *B. globigii* spores irradiated in air at different temperatures demonstrated a marked increase in ultraviolet sensitivity at 4 and 55°C as compared with 24 and 37°C. Thermophilic spores, on the other hand, were only slightly more sensitive at 4 and 75°C than at 24, 37, or 55°C. Representative experiments are reported in Fig. 1 and 2, respectively. Changes in radiation sensitivity at different temperatures are more apparent if the data are reported as in Fig. 3 and 4. To obtain these, a constant ultraviolet dose, *i.e.*, seconds of irradiation, was selected and the number of survivors and mutants were plotted against the temperature

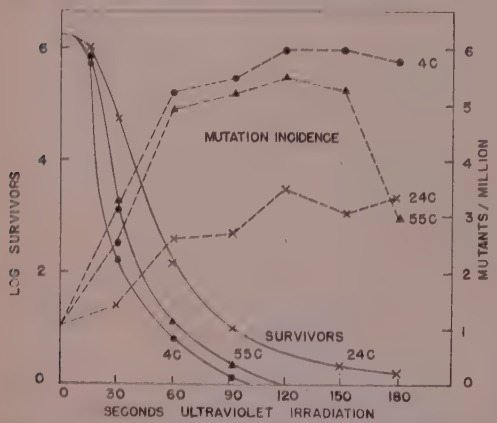

FIG. 1. Survivor and mutation incidence curves for *Bacillus globigii* at different temperatures.

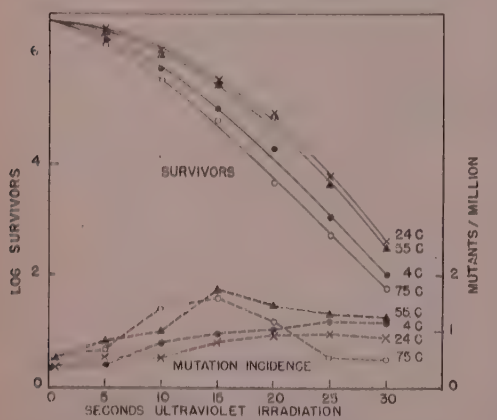

FIG. 2. Survivor and mutation incidence curves for *Bacillus stearothermophilus* spores at different temperatures.

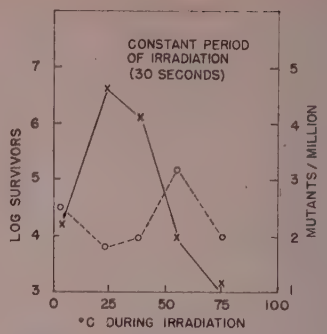

FIG. 3. Influence of temperature upon the effects resulting from aerobic ultraviolet irradiation of *Bacillus globigii* spores. Suspensions irradiated contained 301 million spores/ml. Spontaneous mutation incidence was 1.1 mutants/million (2 μg/ml streptomycin). × = survivors; O = mutation incidence.

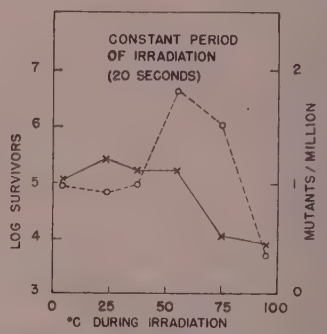

FIG. 4. Influence of temperature upon the effects resulting from aerobic ultraviolet irradiation of *Bacillus stearothermophilus*, Strain 1503, spores. Suspensions irradiated contained 7 million spores/ml. Spontaneous mutation incidence was .5 mutants/million (2 μg/ml streptomycin). × = survivors; O = mutation incidence.

range for which killing and mutability curves had been determined. These points were arbitrarily taken as 30 and 20 seconds, respectively, for *B. globigii* and *B. stearothermophilus*, since the latter is the more sensitive to the lethal action of the radiation. Other time intervals might just as well have been selected, but these lay approximately halfway along the logarithmic portion of the survivor curves. Mutability data may alternatively be reported by plotting the mutation incidence resulting from that ultraviolet radiation dose which yields 99.9% killing. This method gives information which is more suitable for comparison purposes, and such data are reported in Table I.

TABLE I. Influence of Temperature upon Incidence of Streptomycin Resistant Mutants Following an Aerobic Ultraviolet Irradiation Sufficient to Yield a 99.9% Kill of *Bacillus globigii* and *Bacillus stearothermophilus* spores.

Temp. of irradiation	4	24	37	55	75	90
*B. globigii**	2.5†	3	3.1	3.2	2	—
*B. stearothermophilus**	1	1.1	1.4	1.6	1.3	.4

* Incidence of spontaneous mutants: 1.1 and .5 mutants/million, respectively.

† Figures are incidence of end-point mutants in mutants/million.

Comparable information was obtained over a temperature range from 4 to 55°C for log phase vegetative cells of each organism; the temperature effects were much more marked than were noted with spores. Even thermophilic cells became markedly sensitive at 55°C. Comparison at the higher temperatures were not reliable, however, because many of the cells were killed by the action of heat alone.

The high solubility of oxygen in the buffer at low temperatures undoubtedly explains in part the enhanced radiation sensitivity noted at 4°C. The maximum resistance to radiation of the spores of each organism occurred in the vicinity of the optimum temperature for its growth. Temperature controls in each experiment indicated that many of the spores of *B. globigii* were destroyed by the heat alone at temperatures above 75°C, but at lower temperatures viable counts remained constant. The viability of the spores of *B. stearothermophilus* was practically unaffected by heat at any temperature used.

The influence of oxygen upon radiation sensitivity is demonstrated in Fig. 5, which reports data resulting from experiments in which *B. stearothermophilus* and *B. globigii* spores were irradiated in a hydrogen atmosphere at different temperatures. Survivor curves are not reported, since only high temperatures had any effect upon *B. globigii*, whereas, the thermophile was unaffected at the temperatures tested. The data are, therefore, reported in the same way as was done in Fig. 3 and 4. However, it will be noted that with both organisms there was a steady increase in sensitivity to radiation with increasing temperature, but that *B. globigii* became much more sensitive than did *B. stearothermophilus*. There was no increased sensitivity at 4°C, confirming the observation of Hollaender *et al.*(7) that oxygen is required for such an effect. The end-point mutation incidence of the thermophile and mesophile dropped below the spontaneous level when irradiated at 100 and 75°C, respectively, possibly due to some selective factor.

Conclusions and summary. (1) Temperature and atmosphere exert an important influence upon the effects produced by ultraviolet irradiation of the mesophile and thermophile studied. These effects are noted even in the relatively inert spore stage of each. Although the thermophile was more sensitive than the mesophile to the lethal action of a given dose of ultraviolet radiation, it was affected to a lesser degree by changes in temperature during irradiation in the air. Temperature changes exerted almost no effect upon the thermophile when oxygen was excluded from the suspension during irradiation, while the mesophile was markedly affected. This would be the expected result if the thermophile possessed heat-stable nuclear proteins, and damage due to the radiation were not additive with that resulting from heat-denaturation. Mutation incidence at 99.9%

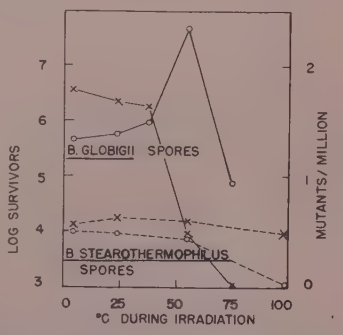

FIG. 5. Influence of temperature upon the effects resulting from anaerobic (hydrogen gas) ultraviolet irradiation of *Bacillus globigii* and *Bacillus stearothermophilus*, Strain 1503, spores. *B. globigii*: 200 million spores/ml-irradiated 30 seconds at each temperature. *B. stearothermophilus*: 8.2 million spores/ml-irradiated 15 seconds at each temperature.

killing fell to below normal levels at 75 and 100°C for the mesophile and thermophile, respectively, possibly due to selection. (2) The relative mutability of the two was also confirmed by the determination of fermentation (mannose) mutants, and by isolation of biochemical mutants using the Davis(12) technic. Such mutants were readily isolated from *B. globigii* cultures, but only a limited number of vitaminless and amino acidless, and no purineless or pyrimidineless mutants could be isolated from the thermophilic cultures. Generally, the thermophile was much less mutabile than the mesophile as compared by streptomycin resistant, fermentation and biochemical mutants.

We wish to express our thanks to Drs. O. B. Williams, and O. Wyss, Department of Bacteriology, University of Texas, for their valuable counsel and cooperation shown the authors during the course of this investigation.

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Depression Red Cell Iron Turnover by Transfusion. (19257)

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One of the commonly employed methods for study of the regulation mechanisms of a steady state is to observe the events which follow an increase or decrease in the regulated quantity.

This paper is concerned with the change in erythrocyte production rate, subsequent to an increment in the red cell volume (RCV) of rats, as measured by plasma and red cell iron turnover using Fe^{59}.* The method for increasing the red cell volume was by intraperitoneal transfusions of blood obtained from the same highly inbred strain.

Hess(1) discovered that rabbits which had been rendered polycythemic by repeated transfusions developed marrow aplasia and fibrosis. Boycott and Douglas(2) contended that only excessive red cell destruction, *not* inhibition of production, explained the disappearance of cells from artificially plethoric rabbits. Although some of their own data would now be considered clearly indicative of lessened production, this concept was not accepted until Robertson's(3) work. Later Krumbhaar(4), Boycott and Oakley(5), and others used the reticulocyte percent as an index of marrow erythrocyte production, and they noted a decline in reticulocytes during the early weeks of transfusions. Although most of the early workers used blood which was initially compatible, several noted development of agglutinins. Possible causes for the inconsistent results in early work will be discussed.

Methods. The iron turnover in plasma and red cells was determined using tracer amounts of Fe^{59}. Plasma iron turnover is the quantity

* No attempt is made here to elaborate on the mechanism through which a change is induced in rate of production by the increased RCV.

of iron entering or leaving plasma per unit time. It is calculated as follows: Plasma Fe turnover (μg/day) $= \frac{0.693}{Fe^{59} \text{ half-time (hr)}} \times$ plasma volume (ml) $\times$ concentration of Fe in plasma (μg/ml) $\times$ 24 (hr). The fraction of tracer present in cells at 3 to 5 days after injection may be used as an index of the portion of turnover in plasma going to marrow and thence to red cells. $\frac{Fe^{59} \text{ in all red cells}}{Fe^{59} \text{ injected}} \times$ plasma Fe turnover $=$ iron entering cells (μg/day). The Fe^{59}, of high specific activity, was combined with globulin prepared from fraction IV-7 (Cohn). The resulting solution had a protein concentration from 5% to 7% and contained 0.73 μc per injection of 0.25 ml. The amount of nontracer iron present in the preparation was negligible.

Twelve female Slonaker rats weighing from 180 to 210 g were used. After preliminary red cell counts and reticulocyte counts, 6 of these rats were given 2 ml of blood 7 times during 6 days. The donor blood from lightly etherized animals *of the same sex and highly inbred strain* was obtained from the inferior vena cava in heparinized syringes and pooled before intraperitoneal injection. Red cell counts and reticulocyte counts were done on the control rats 4 times during the first 9 days and on the experimental rats at the times plotted on Fig. 1. Capillary tube hematocrits were obtained on all animals. On the 8th day,

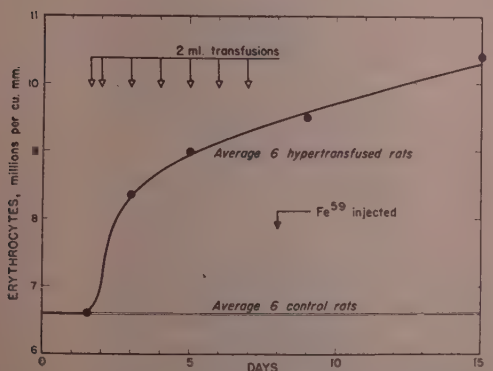

FIG. 1. Erythrocytes, millions/mm³ in control and transfused rats. Controls S.D. $\pm$ 1.7 million/mm³.

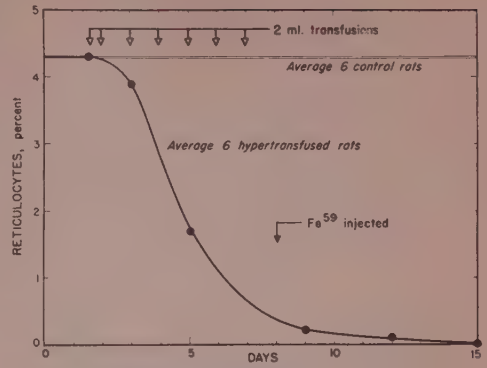

FIG. 2. Reticulocyte decline with increase in red cell volume. Controls S.D. $= \pm 1.6\%$.

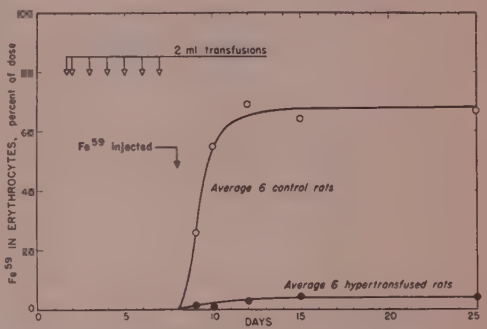

FIG. 3. Percent of injected Fe^{59} in cells. The plateau values were used with the iron turnover in plasma in computation of the rate of iron utilization in erythrogenesis. The red cell iron utilization in the experimental group had diminished to less than 1/10 that of the controls.

one day after the 7th and last transfusion, the Fe^{59} globulin was injected into the tail veins of all 12 animals. Small capillary tube blood samples were taken at 10 minutes, at 3 hours and 50 minutes, and at 10 hours and 48 minutes. After centrifugation, the cell and plasma portions were assayed separately in an efficient gamma fluorescence counter(8). The counting rates per milliliter were computed and plotted as a function of time on semi-logarithmic paper. A line through these points was extrapolated to zero time. The plasma volume was determined by dividing the counts per minute injected by the counts per minute per milliliter at zero time. The total blood volume was computed using the mean hematocrit of the early samples. The red cell volume was found by subtraction. The sam-

TABLE I.

	Plethoric (hyper-transfused)	S.D.	Control animals	S.D.
Avg red cell vol., cc/100 g body wt	5.4		2.53	
Avg hematocrit after transfusion period in %	59.7	± 2.8	40.7	± 3.7
Avg Fe^{59} half-time in plasma (hr)	2.23	± .57	1.58	± .35
% of inj. Fe^{59} in erythrocytes (avg)	4.2	± 3.9	67.6	±12.8
% of inj. Fe^{59} in liver at necropsy	92.9	± 3.5	29	± 3.8
Total plasma iron, μg/ml	5.55*	± .53	3.54*	± .97
" " vol. in ml	5.9	± .82	7.84	± .54
Fe turnover in plasma, μg/day	308		265	
Fe turnover in red cells, μg/day	14.8		177	

* Total iron was determined on plasma removed 17 days after determination of the tracer half-time. Use of this value would be invalid in absolute computation of the turnover but is very useful in comparison between the two groups.

pling was repeated at 24 hours, 53 hours, 4 days, 7 days and again when the animals were killed. Before necropsy the animals were lightly anesthetized with ether; the abdominal cavity was opened; as much as possible of the animal's blood was removed from the inferior vena cava; and samples were taken for Wintrobe tube hematocrits, for red cell radioactivity, and for total plasma iron determinations. The rats, with jugular veins severed, were then perfused with saline until they expired. The livers were excised and total radioactivity of each was determined and expressed as a fraction of the injected radioiron. The red cell volume was used together with the counting rate measurements in calculating the total radioactivity in the red cells.

Results and discussion. Fig. 1 shows the increase in the mean red cell count of the transfused animals. This is in agreement with the previous work(6,7) on transfer of erythrocytes from the peritoneal cavity. The mean of 30 red cell counts in controls during 15 days was 6.6 ± 1.7 (SD) million per cu mm. The rise for several days after stopping transfusions probably results from a readjustment in plasma volume. It is similar to that observed in dogs(4). Fig. 2 indicates the decrease in average reticulocyte percent of transfused rats. The mean reticulocyte percent of the 30 counts on controls was 4.0 ± 1.6 (SD). Fig. 3 expresses the percent of injected iron 59 present in the total red cell volume as a function of time. The differences between the experimental and the control groups are in every detail evidence for a profound decline in production rate in the plethoric animals (Table I). The moderate increase in plasma iron turnover observed in the transfused animals has been associated with red cell-to-plasma transfer increase expected with the normal cell destruction rate of the larger red cell volume.

The normal major pathway, plasma-to-marrow-to-erythrocytes, appears to be greatly diminished with the plethora. The decline in plasma tracer concentration is associated, not with an erythrogenic utilization, but with the transport of nontracer iron from the broken down cells to the iron storage depots. The portion of the plasma iron turnover attributable to red cell production in the plethoric animals was less than 1/10 that of the controls. Radioactivity in the livers of the experimental group was 93% of the Fe^{59} injected whereas only 29% was found in the livers of the control animals. If any liver radioactivity was due to red cells remaining there after the perfusion, it is likely that a greater portion would be present in the control animal livers because of the higher specific activity of their red cells.

The plasma and red cell iron turnover phenomena observed in this experiment are almost identical to those seen in Peruvian natives (14,500 ft.) when they are moved to sea level and studied(9). The shift of tracer from plasma to liver in the hypertransfused animals is similar to that observed by *in vivo* ferrokinetic studies in patients(10,11) who also showed morphologic evidence of marrow hypoplasia.

There are a number of fundamental differences between these experiments and earlier studies. The problem of antibody development is avoided by use of rats whose blood and tissue compatibilities are so complete that parabiotic union can be accomplished and maintained. None of the donors was used more than once.

Experiments at this laboratory in which rats received 4 ml of homozygous plasma per day for 2 weeks, have shown that plasma transfusion does not cause a reduction in red cell production. Others have made the same observation(5).

The inhibition of red cell iron turnover by increasing the red cell volume may be useful in studies of control of erythropoiesis.

Summary. (1) A profound decrease in the red cell iron turnover occurred after experimental increase in the red cell volume of rats. (2) A major portion of the tracer was found in the liver at necropsy.

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Species Specificity of Thromboplastin: Role of the Cothromboplastin Reaction (19258)

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It has been recognized for a long time that species differences may influence the coagulative activity of thromboplastin (tissue extract) on plasma. Thus Quick(1) found that mammalian thromboplastin was relatively inactive with respect to the coagulation of avian plasma and avian thromboplastin was similarly inactive with respect to mammalian plasma. Thromboplastin from rabbit brain was highly active with respect to human plasma but this was not true of thromboplastin from guinea pig brain. Recently Burstein(2) has reported that the activity of mammalian thromboplastin with respect to avian plasma could be greatly increased by the addition of mammalian serum to the reaction mixture: for a maximal effect addition of 0.1 ml of undiluted serum to the 0.3 ml reaction mixture of the prothrombin time test was needed. We have found that normal serum greatly increases the activity of thromboplastin with respect to dicumarol plasma. This effect will occur with highly diluted serum (1:100) provided that the serum and thromboplastin are allowed to react for a brief period before the plasma is added. We have presented evidence (3-5) that this reaction, which we have called the cothromboplastin reaction, is attributable to a specific factor, cothromboplastin, which is present in both plasma and serum and is decreased by dicumarol much more rapidly than is prothrombin. It seemed worth while to determine whether cothromboplastin might be involved in the property which homologous serum has of increasing the activity of thromboplastin with respect to heterologous plasma.

TABLE I. Effect of Dilute Serum on Reactivity of Thromboplastin and Plasma. Thromboplastin pretreated for 3 min with calcium and material tested, then clotting reagent (plasma) blown in. (With important exceptions noted.)

Material tested	Clotting time, sec.
A. Rabbit thromboplastin and chicken plasma	
Saline solution	86
Normal rabbit serum 1-100	20
1-100	47*
1-100	>240 (fibrinogen-acacia instead of chicken plasma)
1-10	14
Dicumarol rabbit serum 1-100	81
1-10	76
B. Guinea pig thromboplastin and human plasma	
Saline solution	76
Guinea pig serum 1-100	15
1-100	37*
1-100	>120 (fibrinogen-acacia instead of human plasma)
Human serum 1-100	85
C. Chicken thromboplastin and rabbit plasma	
Saline solution	68
Chicken serum 1-100	15
1-100	50*
1-20	11
1-20	>1800 (prothrombin-free chicken plasma instead of rabbit plasma)
Rabbit serum 1-100	67
1-20	60

* Not pretreated.

Method. Thromboplastin was prepared from the brains of rabbits, guinea pigs and chickens, by the procedure described by Quick (6). Each brain was dehydrated with acetone. The method of testing thromboplastic activity was essentially the Quick prothrombin time procedure with one additional step. This was a brief period of exposure of the thromboplastin to dilute serum in the presence of calcium, after which the plasma (instead of the calcium as in the usual Quick prothrombin time procedure) was blown in and the clotting time measured. When dicumarol plasma was employed as clotting reagent we have referred to this procedure as a one-stage cothromboplastin assay. It was suitable for testing materials which contain no appreciable quantity of prothrombin; for testing plasma a different procedure was used(3). The serum specimens tested were obtained by allowing blood to clot in small glass tubes and then to stand 24 hours at room temperature. The specimens of plasma were used within 2 hours after the blood had been drawn. The reproducibility of the reported clotting times was of the high order well known to be obtained with the prothrombin time by persons experienced with this test; accordingly the differences in the representative experiments reported are quite gross. One hundred eleven clotting experiments were done in this study.

Observations. Pretreatment with a 1:100 dilution of rabbit serum for 3 minutes greatly increased the activity of rabbit thromboplastin with respect to chicken plasma (Table IA). This brief interval of pretreatment was required for the reaction since there was much less effect if it was omitted. No significant quantity of thrombin formed during this period however, as would be expected since the serum was essentially free of prothrombin. (This was checked by the two-stage method.) Serum from a dicumarolized rabbit had virtually no effect on the activity of the thromboplastin. (The dicumarol serum had somewhat more labile factor activity than the normal serum as judged by shortening of the prothrombin time of aged plasma.)

The relative inactivity of guinea pig thromboplastin with respect to human plasma was corrected by dilute guinea pig serum but not by human serum (Table IB). Similarly, chicken thromboplastin was rendered more reactive with respect to rabbit plasma by

TABLE II. Thromboplastin from Normal Rabbit Brain Compared to That from Dicumarolized Rabbit Brain. Procedure as in Table I, using dicumarol rabbit plasma as clotting reagent. Plasma from same rabbit as dicumarol serum used in Table I.

Material tested	Clotting time, sec. Normal thromboplastin	Clotting time, sec. Dicumarol thromboplastin
Saline sol.	64	190
Normal rabbit serum 1-100	15	15
Dicumarol rabbit serum 1-100	50	

treatment with dilute chicken serum but not by rabbit serum (Table IC). In order to be effective, the serum must be homologous to the thromboplastin, not to the plasma.

Thromboplastin from the brain of a dicumarolized rabbit has been reported to be relatively inactive with respect to dicumarol plasma(7-9). Pretreatment with dilute normal homologous serum removed this difference and greatly shortened the prothrombin time of dicumarol plasma (Table II). These data are included for the purpose of comparing the effects obtained with normal heterologous and with dicumarol homologous plasmas.

Summary. Species specificity of thromboplastin is largely eliminated by brief treatment of the thromboplastin with dilute homologous serum. This appears to be an example of the cothromboplastin reaction.

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Ineffectiveness of Parenteral Pyroxidine in Relieving or Preventing the Egg White Syndrome in the Rat.* (19259)

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The report of MacKay and Barnes(1) that signs of the egg white syndrome were cured in the rat by the injection of vit. B_6 when corn oil was included in the ration, has been interpreted by Sherman(2) in a recent review, to offer evidence that ". . . pyridoxine and essential fatty acids act mutually to enhance or replace biotin."

Salmon and Goodman(3) observed that the severity of the skin lesions in the egg white syndrome were decreased and their character altered somewhat by the presence of linseed oil, butter fat or hydrogenated cottonseed oil in the diet. They attribued this effect to the unsaturated fatty acids which were supplied by the fats. On the other hand, Nielson and Elvehjem(4,5) found that neither corn oil (5 or 10%) nor pyridoxine (100 to 125 μg orally, daily) offered protection against spectacle eye condition or paralysis in their rats on egg white rations. However, as there was the possibility that the effectiveness of pyridoxine in the study of MacKay and Barnes might have been attributable to their use of the parenteral method of administering the vitamin, this was explored.

Experimental. The basal ration had the following percentage composition: egg white

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† A part of the results are from a thesis submitted by Doris Johnson in partial fulfillment of the requirement for the degree of Doctor of Philosophy, University of Wisconsin, Madison.

TABLE I. Effect of Parenteral Administration of Pyridoxine and of Biotin on Manifestations of Egg White Syndrome in the Rat.

Group	No. of rats	Type of exp.	Vitamin inj. mg B_6	Vitamin inj. μg biotin	Severity of symptoms	Days on dose	% of groups C and D showing initial signs of deficiency at ..... days: 17	19	22	27
A		Curative	.2							
	4				3+	0				
	4				3+	14				
	3				3+	21				
	1				4+*	58				
B		"		1						
	8				4+	0				
	8				2+†	14				
	8				0 to 1+†	21				
C		Preventive	.2							
	15				2+		47	47	60	93
D		Control								
	19				2+		33	55	77	100

* Symptoms of the 58th day included: pronounced "spectacle eyes," swollen red bare lips, extensive alopecia and dermatitis, and severe "paralysis."

† Initial symptoms were measurably modified in 1 wk, were inconspicuous after 2 wk of therapy, and at the end of 3 wk there remained only a slight bareness around the eyes of 2 rats and a bristly appearance of the new thick hair.

20, sucrose 71, salt mixture(6) 4, and corn oil 5. The following weights of vitamins were incorporated per each 100 g of ration: 0.2 mg thiamine, 0.2 mg riboflavin, 0.4 mg pyridoxine, 2.0 mg pantothenic acid, and 0.1 g choline. Two drops of a cod liver-corn oil mixture of the following composition were given each rat every 5 days: 45 ml corn oil, 50 ml cod liver oil, 5 g alpha-tocopherol, and 0.4 g methyl napthoquinone.

Moderately pronounced signs of the egg white syndrome were produced in male albino rats in the short period of 35 days, thus giving no evidence that the corn oil of the ration delayed the onset of the symptoms significantly in comparison with earlier experiments in which corn oil was not fed. From the 36th day Group A (Table I) received daily injections of 0.2 mg of pyridoxine hydrochloride. At the end of 14 days, a period which had sufficed for almost complete cure in the rats of MacKay and Barnes, the condition of these animals was fully as pronounced as before; this was also true for the rats which were injected beyond this period (Table I).

Three rats in Group A received, from the 14th or the 21st day, an additional daily supplement namely, a dose of biotin by stomach tube, in an attempt to determine whether or not the injections of pyridoxine would make a measurable difference in the use of biotin by the animals. However, the response (not recorded in Table I) proved to be similar to that obtained in the control rats from similar doses of biotin alone, administered by stomach tube.

In contrast to the lack of benefit obtained from injections of pyridoxine, the curative effect of parenterally administered biotin may be seen in Table I, Group B. To exaggerate the contrast, the symptoms were allowed to become more severe in Group B than in Group A before injections were begun. One μg biotin per day was injected for 21 days, resulting in a rapid regression of the symptoms (Table I). In a more crucial test than the "curative" one above, 200 μg doses of pyridoxine hydrochloride were injected daily into 15 weanling rats from the time that they were first put onto the egg white ration. The record of Groups C and D in Table I shows that there was no appreciable difference between these injected rats and their controls without injections in regard to the time of onset of signs of biotin deficiency. Hence, parenteral doses of pyridoxine failed to enhance or replace biotin for the rat under the conditions of this

experiment in which essential fatty acids were also available.

In attempting to account for the disparity in the results in the two laboratories, one might conjecture that the deficiency symptoms produced by MacKay and Barnes were primarily those of pyridoxine lack. No evidence was presented by them that the symptoms would respond to the administration of biotin. The method of producing the manifestations over so prolonged a period would further leave their interpretation open to question. It has been the experience of several laboratories(7,8) that the sex and initial weight of the rats placed on egg white rations are very important factors in the development of the characteristic egg white syndrome. Male rats show earlier and more marked manifestations than females. Gradations in depletion may also be seen between groups started at 35, 45 and 55 g initial weight respectively; the larger the animals, the more resistant they are to the onset of the characteristic signs of depletion. Hence, it may be significant that the rats of MacKay and Barnes were 40-day-old females with an initial body weight of about 70 g, whereas the rats in the present study were 21-day-old males with an average body weight of 37 g. Inasmuch as the source of pyridoxine in the basal ration of MacKay and Barnes was not the pure vitamin but 5% of yeast, the degree of its availability was not established. The exact requirement would also be conditioned by the amino acids furnished by the basal ration.

Summary. Parenteral doses of pyridoxine failed to cure or to delay the onset of the egg white syndrome in rats on a ration which included the essential fatty acids. The assumption by others, therefore, that ". . . pyridoxine and essential fatty acids act mutually to enhance or replace biotin," is not supported by these experiments with the rat.

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Adrenocorticotrophic Hormone: Stability Studies.* (19260)

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The effects of hydrogen ion concentration, ionic strength and temperature upon the biological activity of adrenocorticotrophic hormone (ACTH) have been investigated in order to define conditions which may be expected to produce little or no inactivation of the hormone. Stability data are obviously of considerable practical importance for the successful isolation of the hormone from pituitary, for the evaluation of its potency by bioassay, and for the preparation of solutions suitable for clinical use. In addition, conditions which inactivate pituitary contaminants and which have little or no effect upon ACTH activity can be applied to the biological puri-

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TABLE I. Stability Studies on ACTH Crude Extracts of Pituitary. Single-Dose Assays. All extracts, 1 g of dry pituitary per 100 ml of extraction fluid.

Exp. No.	Preparation and treatment, hog pit.	Dose in μg	Individual responses*	Avg response	Estimated change in activity, %
1	Extracted with .9% NaCl-.01 N NaOH at 4°C for 10 min	5	143, 93, 123, 99, 102, 125, 100	112	
	Extracted with water at room temp., 3 hr	50	49, –13, –17, –58, 18, 24	1	>–98
2	Extracted with .9% NaCl-.01 N NaOH at 4°C for 10 min	5	116, 101, 118, 86, 195	123	
	Extracted with water at pH 7 at room temp. for 10 min—stored at 4°C, 24 hr	5	–4, 28, 0, 4, –16, –6	1	>–90
	Extracted with water at pH 8 at room temp. for 10 min—stored at 4°C, 24 hr	5	–17, 9, –9, –46, –1, –27	–15	>–90
3	Extracted with .9% NaCl-.01 N NaOH at room temp., 30 min	5	104, 129, 143	125	
	Extracted with water at room temp., 30 min	7.5	–5, 23, –8, –11, 47	9	>–90
4	Extracted with .9% NaCl-.01 N NaOH at room temp., 10 min	5	107, 150, 96, 109, 105, 74, 62	100	
	Same for 30 min	5	87, 94, 80, 95, 30, 14, 119	74	ca-50
	" " 180 min	5	11, 46, 34, –27, 26, 10, 2	15	>–80
5	Extracted with .9% NaCl-.01 N NaOH at 4°C, 10 min	5	103, 191, 160, 136, 79, 191	143	
	Extracted at pH 1 (HCl) at room temp., 10 min—stored 24 hr at room temp.	5	180, 177, 148, 111, 143, 166	154	No sign. change
	Extracted at pH .1 (HCl) at room temp., 10 min—stored 24 hr at room temp.	5	111, 149, 142, 77, 120	120	0 to –50
	Extracted at pH .1 (HCl) at 85°C, 10 min	5	139, 83, 108, 109	110	0 to –50
6	Extracted with .9% NaCl-.01 N HCl, room temp., 10 min	10	103, 198, 166, 120, 124, 116	138	
		2.5	90, 106, 105, 94, 83	96	
	Same extract stored 35 days at 4°C	5	119, 144, 102, 96, 158, 130	125	No sign. change

* Concentration of ascorbic acid in left adrenal minus concentration in right adrenal.

fication of the hormone.

Activation of biological activity must be considered as at least theoretically possible. Li(1) has claimed that potency is enhanced by exposure of a solution of ACTH in weak acid to high temperature. This claim has been subjected to experimental test since it has important implications for practical problems of isolation and bioassay and for theoretical considerations of the chemical nature of the hormone.

Bioassay technic. ACTH activity was determined by the adrenal ascorbic acid-depletion technic in hypophysectomized rats as described by Sayers *et al.*(2). Estimates of potency, based on single-dose assays, are frequently in error by a factor of 2; in a few instances they may be in error by a factor of 4. Single-dose assays have an important role in preliminary screening of potency. For example, complete or nearly complete inactivation of ACTH activity can be correctly evaluated by single-dose assay technic. However, the determination of relatively small changes in activity requires the application of multidose technic. Four to 8 rats at each of 2 or 3 dose levels are employed for standard and for unknown. The estimate of potency with standard error of the estimate is calculated by standard technics(2).

Stability studies. (1) *Crude extracts of pituitary.* The ACTH activity of fresh pituitary remains unchanged for a number of hours after removal of the gland from the animal; the temperature may vary from 4°C to 37°C. However, as soon as cell structure is destroyed, for example, by homogenization, activity rapidly disappears. Fresh frozen pitui-

TABLE II. Stability Studies on Purified Preparations of ACTH. Single-Dose Assays. Concentration of ACTH in solution—20 μg per ml.

Exp. No.	Preparation and treatment, ACTH	Dose in μg	Individual responses*	Avg response	Estimated change in activity, %
7	Sayers *et al.*(3). Sol. in .9% NaCl-.01 N NaOH—assayed immediately	2	137, 102, 87, 191, 86	121	
	Sol. in water at pH 7—stored at room temp., 24 hr	10	30, 20, 0, 21	18	>-95
		2	5, 2, 22, 33	16	
	Sol. in water at pH 7—stored at 4°C, 24 hr	10	83, 36, 38, 29, 37	45	ca-95
		2	38, 9, 28, 10	21	
8	Sayers *et al.*(3). Sol. in .9% NaCl-.01 N NaOH—assayed immediately	1	82, 92, 83, 48	76	
	Sol. in HCl at pH 1—stored at 4°C, 24 hr	1	20, 129, 97, 57, 52	71	No sign. change
9	Sayers *et al.*(3). Sol. in .9% NaCl-.01 N NaOH—stored at 4°C, 24 hr	1	126, 118, 168, 92	126	
	Sol. in HCl at pH 1—stored at 4°C, 24 hr	1	129, 172, 80, 131, 86	120	No sign. change

* Concentration of ascorbic acid in left adrenal minus concentration in right adrenal.

tary rapidly loses ACTH activity when thawed even when the temperature does not rise above 4°C. It appears that ACTH and enzymes which destroy ACTH occupy different loci in the adenohypophyseal cell. Dry pituitary, suitable for the isolation of ACTH, can be prepared from fresh tissue by desiccating the glands in acetone or by lyophilization. Once frozen, the glands should not be allowed to thaw. If fresh frozen tissue is used as starting material for isolation of ACTH, it should be ground in the presence of an extraction medium such as acid acetone in which ACTH activity is stable.

The results of stability studies on crude extracts of porcine pituitary are presented in Table I (Exp. 1 to 6). A 0.9% sodium chloride solution, made to 0.01 N with sodium hydroxide, extracts ACTH activity from pituitary (Exp. 1 to 5). The activity of such an extract is relatively stable at 4°C. However, at room temperature, activity is almost entirely lost after 3 hours (Exp. 4). Sodium chloride exerts a stabilizing effect on purified preparations of ACTH at pH 7.0 or higher. There are no definitive experiments to prove the point, but it is most probable that sodium chloride also exerts the same stabilizing influence on ACTH activity in crude extracts.

Aqueous extracts of pituitary lose ACTH activity very rapidly at room temperature (Exp. 1, 2, 3). ACTH is very stable at pH 1.0 at room temperature (Exp. 5) and at 4°C (Exp. 6). Exposure of ACTH to pH 0.1 for 24 hours at room temperature or for 10 minutes at 80°C may have induced a slight loss in activity although the single dose assays are not accurate enough for definite conclusions on this point. From a practical point of view these stability studies indicate that isolation procedures should be conducted at a low pH. Activity may be expected to be lost rapidly at neutral or alkaline reaction at room temperature; low temperatures diminish but do not prevent loss of activity at high pH values.

(2) *Purified preparations.* Stability studies on preparations of ACTH made by the method of Sayers *et al.*(3) and by the lithium procedure(4) are presented in Table II (Exp. 7 to 9, single-dose assays) and Table III (Exp. 10 to 19, multidose assays).

ACTH is relatively stable in a 0.9% sodium chloride solution made to 0.01 N with NaOH (Exp. 7, 8, 9). In water at pH 7.0 (Exp. 7, 11) and at pH 13 activity is rapidly lost. At high pH values sodium chloride exerts a stabilizing influence upon ACTH activity (Exp. 13, 14). ACTH is stable at pH 1.0 at 4°C (Exp. 8, 9), at room temperature (Exp. 12) and at 100°C (Exp. 15, 16). The resistance of the hormone to destruction by strong acid at 100°C is remarkable (Exp. 17, 18); a 30-minute exposure at boiling tem-

TABLE III. Stability Studies on Purified Preparation of ACTH. Multidose Assays. Standard in solution at pH 1 (HCl).

Exp. No.	Preparation and treatment, ACTH	Conc. ACTH per ml	Estimated change in activity in %, ± stand. error
10	Sayers *et al.*(3). Veronal buffer pH 8.4 at room temp., 24 hr	10 μg	>−90
11	Sayers *et al.*(3). Acetate buffer pH 4 at room temp., 20 hr	10	−15 ±24
	Veronal buffer pH 8.3 at room temp., 20 hr	10	>−90
	Water pH 7.1 at room temp., 20 hr	10	>−90
12	Sayers *et al.*(3). Phosphate buffer pH 7.3 at room temp., 20 hr	10	−27 ±24
	pH 1 (HCl) at room temp., 20 hr	10	−15 ±28
13	Sayers *et al.*(3). pH 12.9 (NaOH) at room temp., 20 hr	10	>−90
	pH 12.6 (NaOH)—.9% NaCl at room temp., 20 hr	10	−82 ±9
14	Sayers *et al.*(3). .9% NaCl at pH 7.2 at room temp., 20 hr		−57 ±21
15	(Lithium). pH 1 (HCl) boiled one hr	20 mg	±13 ±22
16	Sayers *et al.*(3). pH 1 (HCl) boiled one hr	10 μg	−25 ±18
17	(Lithium). 1 M HCl boiled 30 min	20 mg	−37 ±21
18	(Lithium). 1 M HCl boiled 30 min	20	−39 ±9
	Lyophilization	20	+4 ±18
19	(Lithium). Lyophilization.	20	+2 ±17

perature of a solution of the hormone in 1.0 M hydrochloric acid destroys only 40% of activity. Although ACTH is stable in acetate buffer at pH 4.0 (Exp. 11) and in phosphate buffer at pH 7.3 (Exp. 12), it is unstable in veronal buffer at pH 8.4 (Exp. 10, 11).

It is of considerable practical importance to know that lyophilization has no effect upon ACTH activity (Exp. 18, 19). The pH of the medium was adjusted to 4.5 with sodium hydroxide before lyophilization.

Purified preparations of ACTH made by the technic of Sayers *et al.*(3) are precipitated from solution by 10% trichloracetic acid, by 10% tannic acid and by 1% tungstic acid. Single dose assays indicate that the precipitates are biologically active although no exact estimates of potency have been made. Crooke *et al.*(5) have also demonstrated that trichloracetic acid precipitates ACTH activity under certain conditions.

Discussion. The demonstrated stability of ACTH in acid provides a rational basis for the use of acetic acid(6,7) or weak hydrochloric acid(8) for the extraction of ACTH from pituitary. The rapid loss of ACTH activity from neutral aqueous extracts of pituitary suggests that the hormone undergoes enzymatic destruction. It is likely that the pituitary enzymes responsible for inactivation of the hormone are destroyed or inhibited by acid extraction media. The instability of purified ACTH at pH 7 or higher may in part be a result of proteolytic destruction. Indeed, it has recently been found that aqueous extracts of pituitary contain active proteinases (9). However, that instability can not be due entirely to proteinase activity is indicated by recent experiments in this laboratory(10) in which a purified preparation of ACTH was found to be unstable in water following acid heat treatment in 1.0 M hydrochloric acid at 100°C. It is unlikely that pituitary proteinase would survive such treatment. The mechanism by which sodium chloride inhibits ACTH inactivation at neutral or weakly alkaline reaction is unknown.

Weak hydrochloric acid is the medium of choice for the bioassay of ACTH. We use 0.01 N hydrochloric acid routinely in preparing solutions for ACTH assay. This strength of acid exerts no detectable toxic action in the test animal.

The present studies fail to confirm the claim of Li(1) that ACTH activity can be enhanced by exposure of a dilute acid solution to heat. The discrepancy may be due to the fact that Li employed sheep whereas we employed hog ACTH; the strength of acid was not identical. Actually, the results of the single-dose assays presented by Li are not in disagreement with the assay results of this report. It is in the evaluation of the data that the disagreement arises. The error of estimate of the single-dose assays does not indicate that significant activation occurred. Likewise, the

standard multidose technic of bioassay with appropriate experimental design and statistical analysis indicates that weak acid at high temperature induces no significant change in ACTH activity. The single-dose assay is useful for preliminary screening or for detecting relatively large changes in biological activity. However, failure to recognize the error of estimate of single-dose assays can be responsible for incorrect conclusions.

Summary. 1. ACTH activity disappears rapidly from neutral aqueous, 0.9% sodium chloride, or basic extracts of pituitary. Activity is stable in weak hydrochloric acid extracts of the gland. ACTH prepared by the acid-acetone technic(3) or a modification of same (4) is unstable in water; sodium phosphate and sodium chloride exert a stabilizing influence on biological activity. Purified ACTH is stable in weak hydrochloric acid solution. 2. Under the experimental conditions employed we have been unable to confirm the claim of Li that ACTH is "activated" by heating in weak hydrochloric acid solution.

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Adrenocorticotrophic Hormone: Preparation of Material with a High Degree of Biological Purity.* (19261)

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A simple technic has been devised for the isolation of adrenocorticotrophic hormone (ACTH) in good yield. Acid heat treatment of this product results in a preparation with a high degree of biological purity. The product, although chemically inhomogenous, will be of assistance to the clinician and the physiologist interested in determining the therapeutic, toxic and metabolic actions of ACTH rendered free from other biologically active pituitary hormones.

Technic for bioassay of ACTH. The preparations were assayed for ACTH activity by the adrenal ascorbic acid-depletion technic in hypophysectomized rats(1). Potency is expressed as U.S.P. units per mg. One U.S.P. unit equals one International Unit which is the activity of one mg of the International Standard Powder (La-1-A).

The lithium method for isolation of ACTH from pituitary. General principles of the technic. ACTH is soluble and stable in a 1:4 mixture of water and acetone at pH 1.5 whereas 80% of total pituitary solids are insoluble in acid acetone. Extraction of pituitary with this medium effects a 5-fold increase in ACTH activity. At pH 5.6 in the presence of salt, ACTH remains in solution whereas inactive material is precipitated; an additional 2-fold increase in ACTH activity is accomplished by this step. ACTH is recovered by increasing the concentration of acetone in the super-

* This investigation was aided by research grants from the National Institutes of Health, United States Public Health Service. An abstract of part of this work has been published (*Fed. Proc.,* 1951, v10, 189).

CHART 1. Lithium Technic for the Isolation of ACTH. 200 g dried, finely ground hog pituitary tissue—40000 U.S.P. units ACTH activity extracted 3 times with a total of 10000 ml of 80% acetone at pH 1.4 at room temperature.

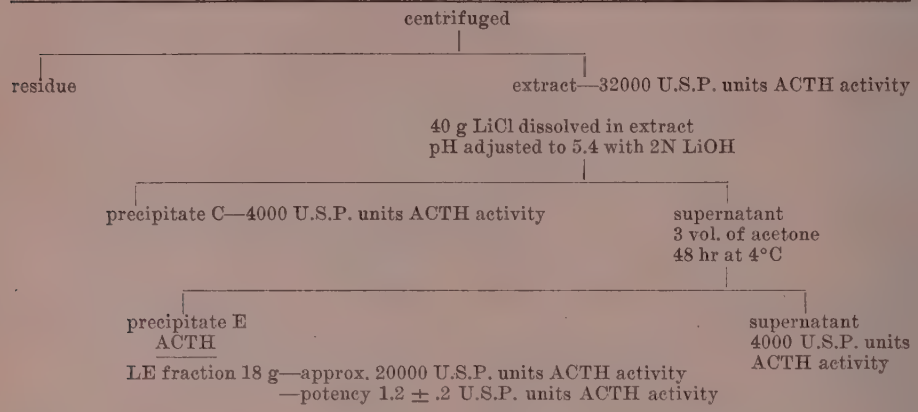

TABLE I. Potency and Yield of ACTH Isolated by the Lithium Technic.

No.	Starting material, g dried pituitary	Total ACTH activity in starting material, U.S.P. units × 1000	Wt LE fraction, mg	Potency of LE fraction, U.S.P. units ACTH activity/mg*	Total ACTH activity recovered in LE fraction, U.S.P. units*
LE-1	10, hog	2	938	.65 to 2	600 to 1900
2	10, hog	2	650	.5 to 2	300 to 1300
3	10, hog	2	900	1 to 1.4	900 to 1300
4	200, hog	40	18000	1 to 1.4	18000 to 23000
6	50, hog	10	2900	.75 to 1.5	2000 to 4500
7	50, beef	2.5 to 5	1500	.75 to 1.5	1000 to 2500

* The limits given in these columns reflect the uncertainty of the assays.

natant to 98%. Both sodium chloride and lithium chloride promote solubility of ACTH in 80% acetone at pH 5.6. However, the lithium salt is preferred since, in contrast to sodium chloride, it is soluble in 95% acetone.

Details of the technic. The following description is for 200 g of acetone-dried or lyophilized, finely ground, porcine pituitary powder. However, any convenient amount of tissue may be employed; the required quantities of the various reagents are proportional to the weight of starting material. Eight liters of acetone (C.P.) are mixed with 2 liters of 0.1 N hydrochloric acid. Four liters of this acid acetone are stirred with 200 g of pituitary tissue for 10 minutes; the pH is adjusted to 1.3 to 1.5 by the dropwise addition of concentrated hydrochloric acid. The mixture is stirred 45 minutes and centrifuged. The extract is collected and the residue is stirred with an additional 4 liters of acid acetone for 30 minutes and centrifuged The residue is extracted a third time with 2 liters of acid acetone for 15 minutes. The 3 extracts are combined, 40 g of lithium chloride are added and dissolved, and the pH is adjusted to 5.4 by the dropwise addition of 2 N lithium hydroxide. A flocculant precipitate forms. An hour later the precipitate is removed on a fluted filter paper. Three volumes of acetone are added to the supernatant, the mixture is stirred and allowed to stand in the refrigerator for 48 or more hours. A gummy precipitate forms and the supernatant liquid is easily siphoned off. The precipitate may be collected and dried by repeatedly washing in acetone or it may be dissolved in water and lyophilized. Chart 1 is a flow sheet of the procedure. Table I presents the yields and potencies of the ACTH preparations which have been obtained by the lithium procedure when applied to both porcine and bovine glands. The yield is approx-

imately 50% of the total ACTH activity in the pituitary powder; the potency is approximately one U.S.P. unit of ACTH activity per mg.

Biological purification of ACTH by acid heat treatment. Effect of acid heat treatment on ACTH activity. A 2% solution of LE-5 in 1.0 hydrochloric acid was exposed to boiling temperature for 30 minutes. Approximately 40% of the ACTH activity was destroyed by such treatment. (See Exp. 18 and 19, Table III of the accompanying paper) (2). It is apparent that ACTH is remarkably stable to acid heat treatment as compared to other adenohypophyseal hormones and posterior pituitary principles. This characteristic of the hormone is the basis of a simple technic for the preparation of biologically pure ACTH. As will be shown below, a 30-minute exposure to 100°C in 1.0 HCl destroys posterior pituitary antidiuretic principle and growth, gonadotrophic and thyrotrophic hormones. Acid heat treatment applied to the LE fraction produces an ACTH preparation with a very high degree of biological purity. Even in the largest doses employed clinically no responses characteristic of posterior pituitary principles or growth, gonadotrophic and thyrotrophic hormones may be expected to occur.

Effect of acid heat treatment on posterior pituitary antidiuretic principle. Antidiuretic activity was measured by inhibition of water diuresis in the rat. The technic employed was that described by Goodman and Gilman (3). Fig. 1 illustrates the results of an experiment designed to determine the effect of acid strength on the destruction of antidiuretic

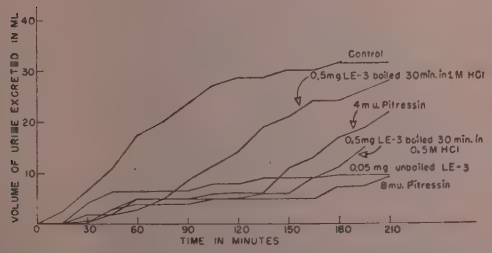

FIG. 1. Effect of strength of acid on degree of inactivation of antidiuretic activity in ACTH preparation.

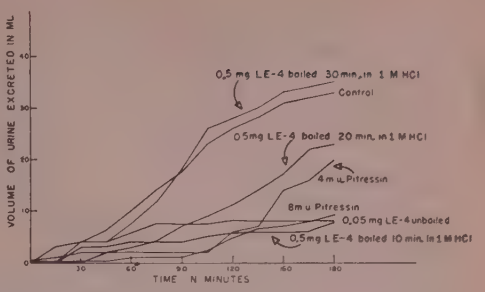

FIG. 2. Effect of duration of heating on degree of inactivation of antidiuretic activity in ACTH preparation.

activity. Solutions of LE-3 (2.0% solids) in 1.0 M HCl and in 0.5 M HCl were placed in a boiling water bath for 30 minutes. Untreated LE-3 in a dose of 0.05 mg and 8 milliunits of Pitressin induced a maximum or nearly maximum inhibition of a water diuresis. Boiling in 0.5 M HCl resulted in a loss of antidiuretic activity. The response induced by 0.5 mg of LE-3, boiled 30 minutes in 0.5 M HCl, was greater than that induced by 4 milliunits and less than that induced by 8 milliunits of Pitressin. This treatment may be estimated to destroy about 90% of posterior pituitary antidiuretic activity. After boiling in 1 M HCl, LE-3 produced a response less than that of 4 milliunits of Pitressin. This treatment is estimated to destroy 98% of antidiuretic activity.

Fig. 2 illustrates the effect of various times of exposure to heat upon posterior pituitary antidiuretic activity. A 10-minute exposure to boiling temperature in 1.0 M HCl had no detectable effect upon antidiuretic activity. A 20-minute exposure resulted in considerable loss (greater than 95% destruction). LE-3, exposed 30 minutes to heat, exhibited no antidiuretic activity; more than 98% of antidiuretic activity was destroyed, a result which confirms the experiment presented in Fig. 1. Each U.S.P. unit of ACTH activity of LE-3 exposed to boiling temperature for 30 minutes in 1.0 M HCl is associated with approximately 0.004 unit of posterior pituitary activity.

Effect of acid heat treatment on growth, gonadotrophic and thyrotrophic hormones. ACTH interferes with the response of the

TABLE II. Growth, Gonadotrophic and Thyrotrophic Activities of LE-5BL and Hog Pituitary Powder. LE-5BL—total dose 50 U.S.P. ACTH units. Pituitary powder—total dose 50 U.S.P. ACTH units. Hypophysectomized adrenalectomized rats—given .9% sodium chloride solution to drink and .1 mg cortisone acetate daily.

	Body wt, g	Testes wt, mg	Seminal vesicles wt, mg	Prostate wt, mg	I^{131} uptake by thyroid % injected dose
Male					
Hypox controls	—16 ± 3	1224 ± 44	52 ± 3.8	14 ± 1.2	1.40 ± .10
LE-5BL	—22 ± 4	1207 ± 56	54 ± 3.5	16 ± .8	1.75 ± .53
Pit. powder	+ 8 ± 6	2954 ± 68	425 ± 14.4	178 ± 7	13.05 ± .69
Female			Ovarian wt, mg	Uterus wt, mg	
Hypox controls	—10 ± 3		33 ± 3.1	74 ± 9.1	2.44 ± .33
LE-5BL	—14 ± 3		30 ± 1.5	86 ± 4.5	2.68 ± .19
Pit. powder	+ 8 ± 2		118 ± 8.4	251 ± 13.9	11.41 ± .91

hypophysectomized animal to growth(4) and to thyrotrophic hormone(5). Furthermore, increased secretory activity of the adrenal cortex interferes with the response of the secondary sex organs to sex steroids(6,7). Hence, an evaluation of the degree of contamination of ACTH preparations with growth, gonadotrophic and thyrotrophic hormones must be conducted in hypophysectomized adrenalectomized rats.

Hypophysectomized adrenalectomized rats were given 1.0% sodium chloride solution to drink and injected with 0.1 mg of cortisone daily.† Hypophysectomy was followed immediately by adrenalectomy. Injections were started 24 hours after operation. The total dose was divided into 30 equal aliquots injected thrice daily for 10 days. The animals were sacrificed the morning after the last injection. The gonads and accessories were weighed. The uptake of I^{131} by the thyroid was measured by a technic described by Ghosh *et al.*(8). The animals were weighed daily during the injection period. Table II presents the results of assay of LE-5BL (30-minute exposure to boiling temperature in 1.0 M HCl) for growth, thyrotrophic and gonadotrophic hormones. The relatively enormous total dose of 50 U.S.P. ACTH units of LE-5BL induced no growth, no increase in weight of the secondary sex organs and no increase in I^{131} uptake by the thyroid, whereas a total dose of 50 U.S.P. ACTH units of dried porcine pituitary induced definite responses characteristic of growth, gonadotrophic and thyrotrophic hormones. The sensitivity of the hypophysectomized adrenalectomized rat may be judged from the observation that a combination of 0.6 mg of a potent growth hormone preparation‡ plus 0.2 mg of thyrotrophin§ plus 5 International Units of pregnant mares' serum produced very definite responses as measured by the 3 indices described above. LE-5BL has a very high degree of biological purity.

Discussion. The lithium method is a simple, inexpensive technic for the preparation of ACTH in high yield. Only three steps are involved in contrast to the large number of manipulations in the method of Sayers *et al.* (9) and of Li *et al.*(10). The low yields of the last two procedures are in large measure due to inactivation as a result of exposure to neutral or alkaline reaction, to losses on dialysis and to incomplete precipitation of ACTH activity by adjustment of pH or salt concentration. The lithium method compares favorably with the glacial acetic acid technic of Payne *et al.*(11).

Exposure of a solution of ACTH in 1.0 M hydrochloric acid to high temperature effectively eliminates posterior pituitary, growth,

† The technic has recently been modified. Hypophysectomized adrenalectomized rats are now maintained with one 15-mg pellet of desoxycorticosterone acetate and given water to drink.

‡ Kindly supplied by Dr. Irby Bundy of Armour Laboratories.

§ Parke-Davis Rx 099802, kindly supplied by Mr. L. W. Donaldson of Parke-Davis and Co.

gonadotrophic and thyrotrophic contaminations. It is likely that acid heat treatment also reduces antigenic activity of the preparation. Undoubtedly, less drastic treatment than that employed in the present studies would still reduce contamination to a clinically acceptable level; this would result in less destruction of ACTH activity and give higher yields. The exact conditions to be employed must await the establishment of limits of contamination of clinically useful preparations of ACTH.

The lithium technic provides starting material suitable for further purification studies on ACTH. LE fractions, obtained from hog and beef pituitary, when processed by the oxycellulose technic of Astwood *et al.*(12), had ACTH activity equal to 10 to 20 U.S.P. units per mg.

Both porcine and bovine pituitary may be employed for the preparation of ACTH. Porcine glands are a potent source of ACTH; each mg of dry porcine pituitary has about 0.2 U.S.P. unit of ACTH activity. In our experience, bovine glands are less potent and more variable in activity; the potency of bovine pituitary has varied from 0.01 to 0.1 U.S.P. unit per mg. Purification of pituitary of sheep has not yet been tried. The one sample of glands of this species which we have assayed by a single-dose test indicated the potency to be about one-quarter that of porcine glands.

The preparations of ACTH obtained by the lithium technic are electrophoretically inhomogenous. Since there appears to be no correlation between biological potency and electrophoretic homogeneity(13,14), no great significance may be attached to this fact.

Summary. A technic has been described for the preparation of ACTH in good yield and in potency acceptable for clinical use. Exposure of a solution of the product in 100°C for 30 minutes results in a preparation with a high degree of biological purity. Hypophysectomized adrenalectomized rats have been employed for the bioassay of growth, thyrotrophic and gonadotrophic hormones which may contaminate ACTH.

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Effects of Cortisone on Electrocardiographic Changes in Adrenal Insufficiency.* (19262)

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(With the technical assistance of Mrs. Sarah Ward.)

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It is a commonly accepted finding in adrenal insufficiency, that functional defects in sodium and potassium metabolism result in a progressive depletion of body sodium reserves and an elevation of the plasma potassium level (1-3). Concomitantly with such alterations in electrolytes, there occur a number of disturbances in cardiac function; among those described have been bradycardia, various arrhythmias, heart block, and decreased cardiac output which eventuate in profound circulatory collapse and terminal cardiac failure(2,4-6). The abnormalities in cardiac function are reflected in electrocardiographic changes which are similar to those seen in other conditions where hyperkalemia is also a prominent finding(7-10). It has been reported(4,5,7) that the electrocardiographic abnormalities seen in adrenal insufficiency are reversed by the administration of sodium chloride or prevented by an adequate intake of sodium. Since cortisone has been found to be effective in correcting the renal functional defect on sodium excretion and in maintaining normal plasma levels of potassium and sodium in the adrenalectomized animal (3), it seemed reasonable that this steroid would have an effect in preventing the appearance of the abnormal electrocardiographic pattern seen in adrenal insufficiency.

With this in mind the experiments described below were done (1) to correlate the electrocardiographic changes in adrenal insufficiency with alterations in plasma levels of sodium and potassium and (2) to assess the effectiveness of cortisone in correcting these abnormalities.

Methods. All experiments were done on bilaterally adrenalectomized dogs. During the immediate postoperative period and in the interval between experiments the animals were maintained on desoxycorticosterone‡ and cortisone. Electrocardiograms and plasma values for sodium and potassium were obtained while the animals were on therapy; the hormone was then discontinued and the animals allowed to go into adrenal insufficiency. During this time serial electrocardiograms were taken and plasma sodium and potassium values determined. When the animals were in a state of severe insufficiency, the effects of intravenous injection of cortisone

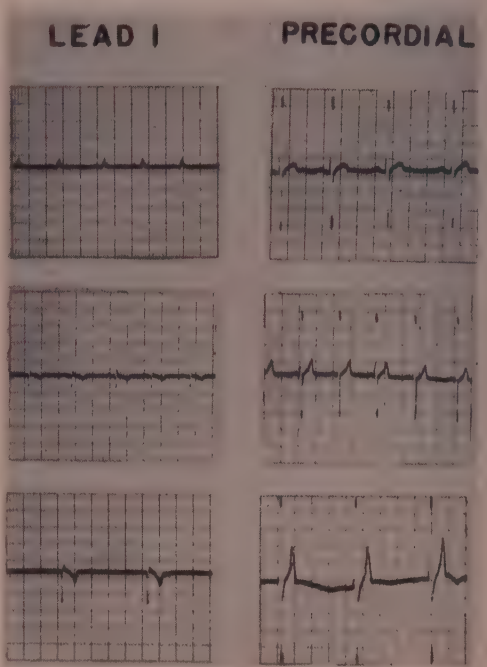

FIG. 1. Progressive EKG changes in adrenal insufficiency. The upper records were taken when plasma values for Na and K were normal. The lower records were taken when plasma sodium level was 129 mM/L and plasma potassium was 7.5 mM/L.

* Aided by a grant from The American Heart Association.

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‡ The author is indebted to the Schering Corporation for the generous supply of Desoxycorticosterone.

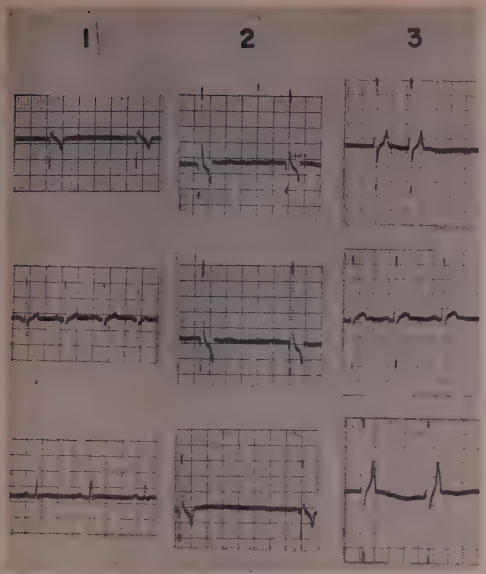

FIG. 2. Effects of (1) cortisone and NaCl on EKG record—lower record taken 3 days after hormone, (2) cortisone alone, (3) NaCl alone—lower record taken 20 hr after NaCl.

and/or sodium chloride on the abnormal electrocardiogram were determined.

Results. Fig. 1 shows electrocardiographic changes as the animals went into insufficiency. Following the withdrawal of hormonal therapy, the plasma sodium level fell and the potassium level rose. The upper record was taken while the animal was adequately maintained with cortisone; the lower 3 records were taken following the withdrawal of the steroid. From these it can be seen that the first alteration seen in the electrocardiographic pattern is an increase in the height and peaking of the T wave; this was frequently seen in the early stages of insufficiency and before any marked changes in sodium and potassium values were evident. With progression of severe insufficiency and marked alteration in plasma electrolyte concentration, there occurred widening of the T wave, bradycardia, disappearance of the P wave and various irregularities in cardiac rhythm.

Effects of sodium chloride. Fig. 2 (No. 3) shows the effects of intravenous administration of .9% NaCl on the precordial lead of the electrocardiogram during severe insufficiency. Within a short time following the administration of saline, the elevated potassium level is lowered and the abnormal tracing is restored toward normal. The beneficial effects of sodium chloride are, however, transient; if no more saline is administered, the animal slips again into exsiccation with a return of the abnormal serum electrolyte concentrations and electrocardiographic pattern within a few hours.

Effects of Cortisone. Although the administration of cortisone partially corrected the alterations in the electrocardiogram, this was true only if the steroid was given before the animals went into severe insufficiency and before marked alterations in plasma sodium and potassium levels had occurred. Cortisone had little or no immediate effect, however, on the electrocardiographic abnormalities if given at a time when the plasma sodium was already severely depressed and plasma potassium elevated. Fig. 2 (No. 2) shows the ineffectiveness of cortisone when given at such a time. However, if the plasma sodium level is restored by administration of sodium chloride and the animal is given cortisone at the same time, as shown in (No. 1) in Fig. 2, not only is the abnormal electrocardiogram restored toward normal within 2 hours but a more nearly normal pattern is maintained for 3 days without further therapy. Comparison of these results with those seen following NaCl shows that the effects of NaCl alone are transient; whereas, cortisone if given with saline will maintain the normal pattern for a longer period of time. Furthermore, it was found that adequate daily maintenance with cortisone, without benefit of added sodium, was effective in maintaining a normal electrocardiographic pattern and normal plasma sodium and potassium values.

Discussion and conclusions. The changes in the electrocardiogram in adrenal insufficiency were found to parallel the alterations in plasma sodium and potassium; these were prevented with adequate maintenance dosages of cortisone but not corrected completely by this steroid after marked abnormalities in the electrocardiogram had occurred. This is in keeping with the concept (2,6) that the defects in cardiac function, found in adrenal insufficiency are the result of losses of body sodium

and elevation of plasma potassium with a consequent imbalance of these ions. If this were the cause, it would be expected that abnormalities of the electrocardiogram would be reversed toward normal only by restoration or maintenance of adequate concentrations of sodium and potassium; such was found to be the case in our experiments.

It is currently taught that the difference in potential which exists across the cell membrane is dependent, in part, upon the difference in concentration of potassium between the intra- and extracellular compartments. Alterations in this concentration, as are seen in conditions of hyperkalemia, result in a variety of disturbances in neuro-muscular activity and, more particularly, myocardial function (9,11,12), which result in such clinically overt findings as described above.

In adrenal insufficiency it has been repeatedly observed(4,5,7), that sodium chloride administration in adequate amounts will lower plasma potassium concentration and restore the electrocardiographic abnormalities toward normal; from this it would appear that these disturbances are directly related to abnormalities in sodium and potassium metabolism and are reversible only if adequate concentrations of these ions are restored.

Since cortisone has been found to correct the renal functional defect on sodium excretion and to maintain normal plasma concentrations of sodium and potassium, it is conceivable that the disturbances in the electrocardiogram seen in adrenal insufficiency are effected by this steroid only in so far as it corrects the abnormalities in electrolyte metabolism.

That this is true under the conditions of these experiments is evident from the finding that cortisone is without effect when marked abnormalities in the plasma concentration of these ions has already occurred; but adequate maintenance with or administration of this steroid early in adrenal insufficiency, without added sodium, is effective in maintaining a normal electrocardiogram.

The author is grateful to Dr. Robert F. Pitts for his kind suggestions and advice concerning this work.

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Hepatic Fibrosis Produced by Chronic Ethionine Feeding.* (19263)

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Ethionine (α-amino-γ-ethylthiobutyric acid), an analogue of methionine, probably inhibits protein synthesis, since it has been shown to inhibit the incorporation of radioactive methionine into glycine(1). It produces within 24 to 48 hours a fatty infiltration of the liver in female but not in male rats(2,3).

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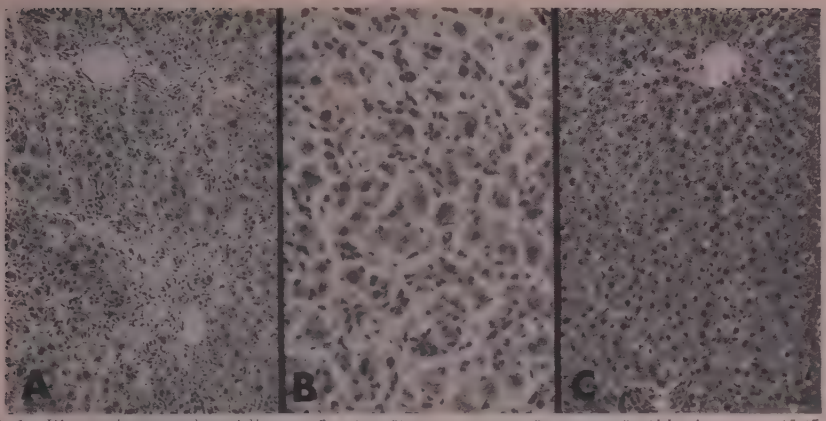

FIG. 1. Photomicrographs of livers of rats after prolonged feeding of ethionine. A. (165 ×). The lobular architecture is distorted and connective tissue septa start to connect central fields. The liver cells in the center of the lobule have disappeared and the liver cell plates are irregularly arranged. The interlobular connective tissue is increased. B. (330 ×). Small groups and individual liver cells revealing differences in size and staining qualities are surrounded by connective tissue membranes which contain fibroblasts, inflammatory cells and proliferated cholangioles. C. (165 ×). With methionine. The lobular architecture is intact. The liver cells show moderate variations in staining qualities of nuclei and cytoplasm.

This is prevented not only by glucose, but also by an amount of methionine more than equivalent to that of ethionine(4). The question arose as to what liver changes are produced by chronic ethionine administration.

Material and methods. Wistar strain white female rats were divided into 3 groups and one group of male animals was added. All animals were on a basal diet, containing 16% casein. All but one group received 0.5% ethionine in their synthetic diet. The daily intake and the weight of the animals were recorded. To exclude any changes possibly caused by the relatively low food intake, when ethionine is added, one female group was pair-fed without ethionine. One group received in addition to 0.5% ethionine, 1.0% methionine in the diet. All animals were killed after 28 days, the histological picture of the liver evaluated, and the total lipids of the liver determined gravimetrically after extraction with chloroform and re-extraction with petrolether.

Results. The livers of the pair-fed animals without ethionine showed slight fatty metamorphosis without fibrosis or other liver damage. The livers of the rats, males and females alike, which received ethionine showed a pale granular outer and cut surface and were of increased consistency. Under the microscope, the lobular architecture appeared somewhat distorted in at least part of the liver. In addition, the arrangement of the liver cell plates was irregular (Fig. 1A). The liver cells varied from very small, atrophic or degenerated to large, regenerating ones. Especially in the latter, the nuclei were rich in chromatin and often contained several large, strongly acidophilic nucleoli. The cytoplasm was usually dark, irregular stained and appeared homogenous in some necrobiotic cells. In the center of the lobule the liver cells had often disappeared. In the rest of the lobule single liver cells or small groups of them appeared separated from each other by connective tissue membranes of variable thickness. These revealed uniform fine reticulum fibers as well as many mesenchymal cells, apparently fibroblasts, and also proliferating cholangioles. Only rarely were the membranes condensed to thicker septa dissecting the lobular architecture. In these septa some of the remnants of sinusoids appeared as wide vessels. Brown, granular pigment was found in epithelial and mesenchymal cells, especially near the center. It was not fluorescent and failed to give iron reaction. Few fat droplets were found in isolated liver and Kupffer cells (Fig. 1B). The

TABLE I. Results of Acute and Chronic Ethionine Administration, With and Without Methionine Supplements, upon Histologic Changes and Total Liver Lipids in Rats.

No. of rats	Sex	Ethionine	Duration, days	Methionine, % of diet	Wt loss in %	Liver: Degree of liver damage	Liver: Histologic fat	Total lipids, g/100 g wet wt
7	♀	—	2§	—	5.6 ± .6	0	0	5.3 ± .2
2	♂	—	2§	—				
12	♀	+†	2§	—	8.8 ± 1.1	0	+++	19.4 ± 1.7
6	♂	+†	2§	—	6 ± .8	0	0	6.2 ± .2
11	♀	+‡	28	—	24.5 ± 4.8	+++	0	3.1 ± .7
7*	♀	—	28	—	10.4 ± 1.6	0	+	7.8 ± 1.8
8	♀	+‡	28	1	9.6 ± 1.9	+	0	6 ± .8
7	♂	+‡	28	—	17.8 ± 3.3	+++	0	3.3 ± .6

* Pair-fed to previous group. † 100 mg/100 g body wt intraper. ‡ .5% of diet.
§ Fasting.

pancreas in these animals showed atrophy of the acini and fibrosis of the interstitial tissue with infiltration by lymphocytes and histiocytes.

The livers of the rats which had received supplementary methionine were grossly normal. Microscopically, the arrangement of the liver cell plates was slightly irregular. The liver cells in the central zone were vacuolated and, in the peripheral zone, they were larger and darker, probably due to regeneration. Around the widened portal triads some proliferation of the cholangioles, fibrosis and pigment deposition was noticed. Without specific location in the lobule, isolated cells or groups of cells revealed many small fat droplets (Fig. 1C).

After chronic ethionine administration, less than the normal amount of liver lipids was extracted chemically and at the same time, the normal body fat depots had disappeared in both sexes while in the pair-fed controls and methionine-supplemented rats the normal amount of lipids in the liver and the fat depots was preserved. For comparison results obtained 48 hours after the intraperitoneal administration of ethionine(2) were included (Table I).

Comments. The extensive fibrosis which is observed after chronic administration of ethionine is of predominantly intralobular location, distorting only slightly the lobular pattern. It represents an unusual type of cirrhosis. The proliferation of the cholangioles reveals some similarity with the lesion produced by butter yellow. So far, however, the marked distortion of the lobular pattern, massive necrosis and excessive regeneration which precede carcinoma formation in the latter, have not been observed. Whether the severe liver cell damage with the associated regeneration is the primary cause of the fibrosis is as yet unproven. It should be stressed that the chronic administration produces not only no increase in liver fat, but oddly enough, a decrease. This decrease parallels the depletion of the entire fat depots of the body and suggest a basic, so far not understood effect of ethionine administration upon the lipid metabolism. The analogy to the gradual transformation of the human fatty cirrhotic liver into a fat-free scarred organ deserves emphasis despite the rapidity of the experimental process. Sex difference in the response to ethionine found in the acute fatty liver(2) and in kidney changes(5) is not present in the chronic lesion here presented and in the pancreatic necrosis and inflammation recently reported and confirmed(6-8). In common with these acute lesions, the chronic hepatic fibrosis can be prevented by simultaneous administration of methionine. These findings suggest that, like the acute damage produced by ethionine, the hepatic fibrosis is an expression of a chronic interference with the utilization of methionine. Nevertheless, to date, a specific toxic effect of ethionine, ameliorated by methionine, cannot be entirely excluded.

Summary. Oral administration of ethionine produces severe liver cell damage and an intralobular type of hepatic fibrosis with reduction of the fat depots of the entire body and of the

normally present fat in the liver. The lesion is prevented by simultaneous administration of methionine.

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Potentiation by Adrenaline of Protective Effect of Cortisone* on Histamine Toxicity in Adrenalectomized Mice. (19264)

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It is well known that mice are relatively insensitive to histamine. According to Halpern and Wood(1) the lethal dose of histamine dihydrochloride, injected intraperitoneally, is for normal mice 50 mg per 20 g‡ of body weight. Adrenalectomy renders the mice considerably more sensitive to histamine so that it requires only 0.5 mg per 20 g of histamine to kill the adrenalectomized mice (2). It has been shown by the same authors that pretreatment with Promethazine increases the resistance of the adrenalectomized mice to histamine and brings it up to that of the normal mice. In the same conditions adrenaline increases also the resistance of the adrenalectomized mice to histamine, but in this case the toxicity of histamine is not reduced to normal; the lethal dose of histamine is brought up from 0.5 mg to 5 mg per 20 g. Adrenaline therefore does not seem to be the only factor responsible for the high resistance of normal mice to histamine.

It is the purpose of this study to investigate whether cortisone increases the resistance of adrenalectomized mice to histamine. It has been reported by various authors that cortisone can protect this species of animals against anaphylaxis(3,4). It should be noted, however, that cortisone does not protect other species of animals either against histamine or anaphylactic shock(5-7).

Experimental. Technic. 112 adult mice (female) weighing about 20 ± 5 g were used. Bilateral adrenalectomy was performed under light ether anesthesia and the animals allowed access to normal diet with plenty of fluid. Right after the operation, the animals received subcutaneously 250 γ of desoxycorticosterone, and 0.5 ml of isotonic saline. The animals treated with cortisone were injected with 0.5 mg of the drug twice a day for 3 doses, the last dose was given 4 to 5 hours before the injection of histamine. The doses of histamine dihydrochloride were administered intraperitoneally in 0.5 ml of normal saline per 20 g of body weight 48 hours after the operation. In some animals, hemoconcentration was measured by the change of the hemoglobin concentration of the blood. Blood samples were obtained by puncture of the ophthalmic venous plexus through the inner anode of the eye with a capillary glass pipette (8), before injection and again 20 minutes after injection of histamine. A group of adrenalectomized animals treated with cortisone was also treated with adrenaline. To these

* We wish to acknowledge the help of Merck who supplied us graciously through l'Institut National d'Hygiène with Cortisone used in this experiment.

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‡ It is considered that the average weight of an adult mouse is 20 g.

TABLE I. Effect of Cortisone and Adrenaline on Histamine Toxicity in Adrenalectomized Mice.

Histamine, mg/20 g	Control animals		After cortisone		After cortisone and adrenaline	
	Mortality	Avg haemo., %	Mortality	Avg haemo., %	Mortality	Avg haemo., %
.25	3/9	31				
.5	5/5	65	0/10	12		
2.5			5/14	31		
5			10/15	50		
10					0/6	2
25					2/15	8
50					7/7	

TABLE II. Effect of Cortisone and Adrenaline on Histamine Toxicity in Normal Mice.

Histamine, mg/20 g	Control animals, mortality	After cortisone, mortality	After cortisone and adrenaline, mortality
50	10/10	7/10	10/11

animals 20 γ per 20 g of adrenaline were injected subcutaneously 30 minutes before the injection of histamine. The results of these experiments are presented in Table I. The effect of cortisone and of adrenaline on histamine toxicity in the normal mouse was also investigated using a small number of animals. The results of these experiments are presented in Table II.

Results. The data presented in Table I show that cortisone can increase from 0.5 to 2.5 mg per 20 g the tolerance of adrenalectomized mice to histamine without restoring to the animals the ability to withstand doses of histamine tolerated by the normal mouse. However, treatment with both adrenaline and cortisone enables adrenalectomized mice to withstand the same doses of histamine as normal mice. The degree of tolerance to histamine afforded by these drugs is reflected in the degree of hemoconcentration. As the animals are protected with cortisone, or cortisone and adrenaline, the degree of hemoconcentration usually caused by the injection of histamine diminishes. Thus, the degree of hemoconcentration observed in adrenalectomized control animals which received 0.5 mg per 20 g of histamine was 65%. In animals treated with cortisone, the same dose of histamine produced an increase of hemoconcentration of 12%; when treated with cortisone, a dose of histamine as high as 2.5 mg per 20 g determined in the adrenalectomized mice an increase of hemoconcentration of 31%. However, after pretreatment with cortisone and adrenaline which enables the mice to tolerate as high a dose as 25 mg per 20 g of histamine, the average hemoconcentration observed is only 8%. The data presented in Table II show that neither cortisone nor cortisone and adrenaline afford much protection to the normal mouse against histamine toxicity.

Discussion. The data presented show that cortisone affords some protection to the adrenalectomized mice against histamine toxicity; the treated animals can tolerate a dose about 5 times larger than the control animals. In this respect cortisone behaves much like adrenaline alone for these adrenalectomized animals. None of these substances can alone restore normal tolerance to a single injection of histamine as is done by promethazine. However, treatment by adrenaline in animals receiving cortisone for 24 hours confers the ability to the adrenalectomized mice to withstand the high doses of histamine tolerated by normal mice. What may be the mechanism of this phenomenon? Levine and his co-workers (8) have observed with adrenalectomized animals that treatment with cortisone increases the vascular effect of nor-adrenaline. According to these authors, cortisone improves the ability of the vascular bed to respond to vasoconstrictor agents.

The experimental data reported here indi-

cate that the decreased tolerance to histamine of the adrenalectomized mouse is directly related to the increase of hemoconcentration caused by the drug. It appears that the angiotoxic effect of histamine is potentiated by the removal of the adrenals. But, as the animals are protected with cortisone and adrenaline, they show a lower degree of hemoconcentration when injected with histamine. It seems that these hormones protect the animals by increasing the resistance of the vascular bed to the toxic effect of histamine on small vessels. This interpretation is consistent with the results obtained either with adrenaline alone, or with the anti-histaminic drugs, which, as it is admitted by certain authors(10), have a definite effect on the small vessels. The fact that neither cortisone nor cortisone and adrenaline afford any protection to the normal mice is consistent with the results obtained with promethazine. In the normal mouse, the toxicity of histamine may be due to a different mechanism.

Summary. (1) When injected into adrenalectomized mice, cortisone enables these animals to tolerate 5 doses of histamine, which are usually lethal for the adrenalectomized animals. (2) Under the same conditions, adrenaline enables adrenalectomized mice to tolerate 5 to 10 lethal doses of histamine. When, however, adrenalectomized mice are treated with both cortisone and adrenaline, they are able to tolerate 50 to 100 lethal doses of histamine, and their resistance to the drug is brought back to the normal level. (3) The relationship between increased tolerance to histamine and lowering of the hemoconcentration effect of histamine caused by cortisone and adrenaline in adrenalectomized mice suggests that these hormones exert their protective effect against the angiotoxic action of histamine on the small vessels. (4) Contrasting with this remarkable action of cortisone and adrenaline in adrenalectomized mice, these hormones do not modify the toxicity of histamine in normal animals.

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Choline Oxidase Activity in Young Rats.* (19265)

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The low activity of choline oxidase in several rat liver tumors(1-3) as compared with normal rat liver, made it of interest to study the activity of this enzyme in the livers of young rats. The succinoxidase system, which is chiefly associated with the large granule (mitochondrial) fraction of rat liver, had been shown earlier(4) to increase rapidly from low levels in newborn rats to the normal adult level in 12-15 days. As choline oxidase has also been shown to be largely associated with the large granule fraction of rat liver(5), in the present study both succinoxidase and choline oxidase activity were measured. The results obtained with succinoxidase are in agreement with the earlier study(4). Choline

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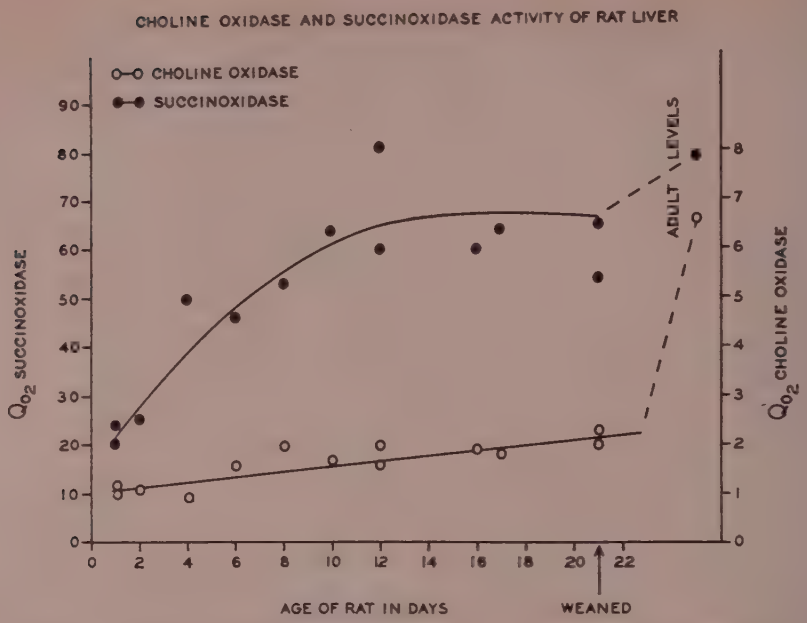

FIG. 1. Choline oxidase and succinoxidase activity of rat liver in unweaned rats.

oxidase activity was found to be low at birth, and at the time of weaning (ca 21 days) attained only one-third of the activity of normal adult rat liver.

Methods. Rats of the Wistar strain, inbred in our laboratories for seven years, have been employed in these studies. The colony is maintained on a Dog Chow ration. The young rats were weaned at 20-22 days of age, usually 21 days. The liver of the rats was assayed for succinoxidase and choline oxidase activity at varying periods after birth. Succinoxidase activity was measured manometrically(6) with the exception that Al Cl_3 was not added. Choline oxidase was measured manometrically as described previously(5). The Q values were calculated in all cases using the assumption that the dry weight of rat livers represents 30% of the wet weight. A few measurements, under similar conditions, of the choline oxidase activity of rat kidney cortex, were also made.

Results. The data obtained on the choline oxidase and succinoxidase activity of the livers of rats from one to 21 days of age are summarized in Fig. 1. Each point represents the value obtained on pooled livers from two to eight animals. The succinoxidase activity, in agreement with Potter, Schneider and Liebl (4), increased rapidly and values in the normal range were obtained by 10-12 days after birth. However, with our animals, the average succinoxidase activity of 32 rats from 10-27 days of age was 20% lower than the adult average. As is shown, choline oxidase activity increased at a much slower rate, attaining at weaning approximately one-third of the adult level. The adult levels represent the values obtained on 27 rats weighing between 200 and 400 g and are shown in Fig. 1.

Choline oxidase activity in the normal adult range was observed when the rats attained approximately 40 days of age, although there was considerable variation among different litters. Small animals were observed to have lower activity than large animals from litters of the same age. The choline oxidase activity of the livers of 95 weaned rats was measured as a function of their age and body weight. The data illustrating the relationship between activity and body weight are plotted in Fig. 2. Each point, except where indicated, represents a single rat. The values on the pre-weanling rats, for which weight as well as age had been

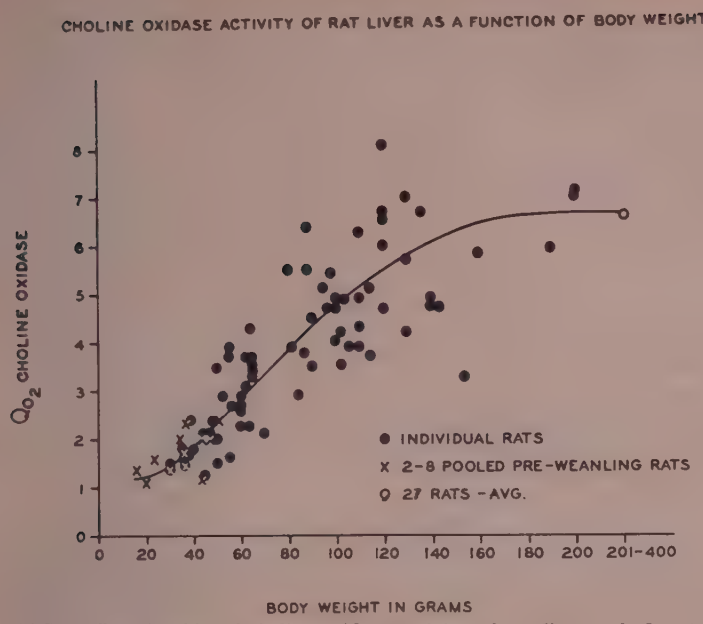

FIG. 2. Relationship of choline oxidase activity of rat liver to body wt.

recorded, are also included for comparison. It can be seen that the activity of choline oxidase in rat liver increases as a function of the body weight reaching maximal values when the animals weighed 120-150 g.

The choline oxidase activity of rat kidney cortex has also been measured in several animals. In adult animals this tissue ($Q = 3.5$) yields approximately one-half as much activity as liver. The activity of the kidney cortex from 8-day-old rats (weight 23 g) was one-seventh of this ($Q = 0.5$) while the tissue in 22- and 23-day-old rats (weight 45-56 g) was one-third ($Q = 1.1$) of the adult value.

Discussion. Although both succinoxidase and choline oxidase activity are associated with the large granule (mitochondrial) fraction of rat liver, the data presented indicate that these two enzymes increase in activity in the livers of young rats at widely different rates, choline oxidase activity increasing much more slowly. The choline oxidase activity of rat kidney cortex has also been observed to be low in newborn rats and to be low at weaning. Presumably the diet used was adequate in all nutritional factors, however, the possibility that the slow rate of increase in choline oxidase activity may be due to a limiting nutritional factor, is under investigation.

The slow increase in choline oxidase activity in young rats may be related to the high requirement for choline or preformed methyl groups by the weanling rat, although a low level of choline oxidase activity would be expected to preserve for other purposes such choline as was available to the animal. The apparent necessity for choline to be oxidized to betaine(7,8) in the rat before its available methyl group can be utilized in transmethylation reactions, suggests the need for data on the activity of other enzymes concerned with labile methyl synthesis and utilization in weanling rats before the significance of low choline oxidase activity may be properly evaluated. Preliminary experiments indicate that dimethylthetin-homocysteine and betaine-homocysteine transmethylase activity do not parallel choline oxidase activity.

Summary. (1) Choline oxidase activity is low in the livers of newborn rats and increases to only one-third of the adult level by weaning, in contrast to the rapid attainment of adult levels of succinoxidase activity. (2) Choline oxidase activity is low in the kidney

cortex of newborn rats and also rises to adult levels very slowly. (3) The slow increase in the choline oxidase activity of rat liver as a function of the weight of the animal is demonstrated.

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Surgical Anesthesia in Rabbit and Dog with Intravenous Amphenone B.* (19266)

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We have previously reported that Amphenone "B"(1) has a potent depressant action on the central nervous system of the rat (2). We now report the experimental use of intravenously administered aqueous solutions of this compound to induce complete surgical anesthesia in the rabbit and dog.

Female New Zealand White rabbits and small female mongrel dogs were maintained on a stock diet. Food was removed the evening preceding operation. No preoperative medication was employed. Either a 2% or 4% aqueous solution of Amphenone "B" was employed. All injections were given by intermittent intravenous infusion beginning with a sufficient volume to induce prompt anesthesia. The surgical procedures listed in Table I were immediately performed. Supplemental doses of drug were administered intravenously as required for maintenance of surgical anesthesia.

The behavior of these animals during the course of these observations warrants the conclusion that Amphenone "B" possesses a sufficiently profound depressant action on the central nervous system to abolish pain response in the rabbit and dog. The accompanying respiratory depression is sufficiently limited to afford a substantial margin of safety in the performance of major surgical procedures. Complete relaxation of the abdominal muscles was noted during each operation. Visceral manipulation such as traction on the uterus and exterioration of the bladder and intestines was carried out with ease and without significant effect on respiration or on the depth of anesthesia. The blood retained a normal oxygenated appearance and only the expected amount of blood loss was observed.

The rate of recovery was recorded as the length of time required for each animal to spontaneously assume an upright position. The speed with which such recovery occurred suggests that the compound is rapidly cleared from the blood.

In the rat Amphenone "B" effects a marked enlargement of the thyroid and adrenal and exerts an atypical folliculoid action on the female genital tract(3-5). In the rabbit it exhibits a weak progestational action on the uterus(4). A number of steroid compounds, notably progesterone, have anesthetic properties when administered in high dosage either intraperitoneally or intravenously(6). Accordingly, the association of anesthetic and endocrine effects in a non-steroid compound such as Amphenone "B" presents a striking parallel to the biological phenomena observed with steroids.

Summary. Intravenously administered Amphenone "B" produces surgical anesthesia in the rabbit and dog. The margin of safety is

* 1,2 bis-(p-aminophenyl)-2-methylpropanone-1 dihydrochloride.

† National Institutes of Health, Public Health Service, Federal Security Agency.

TABLE I. Surgical Procedures Under Amphenone Anesthesia.

Animal	Body wt (kg)	Dose for induction (mg/kg)	Length of induction (min)	Total dose (mg/kg)	Duration of total injection (min)	Duration of anesthesia (min)	Surgical procedure*	Length of operation† (min)	Recovery (min)
Dog 1	10.15	118	5	197	22	70	1	30	140
2	2.39	119	4	218	26	46	1, 2	24	49
3	3.5	230	4	320	27	34	1	17	82
Rabbit 1	1.98	150	3	200	4	16	1, 2	11	16
2	2.20	153	4	254	7	9	1	9	19
3	1.69	118	3	213	7	13	1	7	39
4	2.82	84	3	200	5	17	1	10	32
5	3	195	4	293	11	20	1	12	—

* 1 = bilateral ovariectomy; 2 = subtotal hysterectomy.
† = time from incision to closure. — = not observed.

sufficient to permit complete anesthesia without major respiratory depression and with rapid recovery. The combination of endocrinological and anesthetic properties of Amphenone "B" parallels that previously observed for progesterone and other steroids.

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Effect of Amphenone "B" on Adrenal, Thyroid and Testes.* (19267)

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Recent work by Allen, Hertz, and Tullner (1,2) has shown that when Amphenone "B"‡ (3), a substituted desoxybenzoin, was injected subcutaneously into female rabbits or given intragastrically to female rats, it produced marked thyroid and adrenal enlargement as well as progestation. The adrenomegalic effects were prevented by the administration of cortisone without reducing the goitrogenic effect and, conversely, the administration of thyroxine inhibited thyroid enlargement without affecting the adrenal enlargement (4).

These observations, and the fact that Amphenone "B" did not cause enlargement of the adrenal and thyroid in hypophysectomized animals, suggested that "These responses were brought about through a pituitary mechanism similar to that by which thiouracil induces thyroid hypertrophy" (5). Whereas, the evidence indicates that Amphenone "B" increases the secretion of ACTH and TSH, the data available do not preclude the possibility of an increased production of thyroid and adrenal hormones rather than a decreased production, as would be expected in the case of a blocking agent such as thiouracil. We have extended the foregoing studies in an effort to explain more fully the alterations of thyroid and adrenal size and function and are reporting the results in this paper.

Experimental. Sprague-Dawley rats were

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† This work was done during the tenure of a Research Fellowship of the American Heart Association.

‡ 1,2-bis-(p-aminophenyl)-2 methyl propanone-1 dihydrochloride, kindly supplied by Dr. E. J. Fellows of the Smith, Kline and French Co., Philadelphia, Pa.

used throughout. Two types of experiments were conducted, one relatively chronic and one acute. In the chronic study, 22-day-old male rats (34-44 g) were injected subcutaneously with 5 mg of Amphenone "B" in 0.5 ml of saline at 8:00 A.M. and 5 mg in 0.2 ml of sesame oil at 7:00 P.M. (25 mg per 100 g body weight daily) for a period of 11 days. Eight rats were given Amphenone "B" and 12 served as controls. Twenty-four hours before sacrifice all animals were given 50 μc of I^{131} subcutaneously. Approximately 15 hours after the last dose of Amphenone "B" in oil, the animals were anesthetized with nembutal and immediately before death the adrenal glands were removed, weighed and one gland placed in 30% KOH. Blood was drawn from the aorta for eosinophile counts(6). The thyroids were then removed, weighed and one lobe placed in *2N* NaOH and finally, thymi and testes were removed and weighed. The remaining lobe of the thyroid, the other adrenal gland and the testes were placed in formalin prior to sectioning and staining. Adrenal cholesterol determinations were carried out using a modification of the Schoenheimer-Sperry method(7). The thyroid tissue was homogenized to facilitate dissolution in the NaOH, aliquots pipetted onto planchets and the radioactivity determined by means of a Geiger-Müller counting system. In an effort to determine the effects of Amphenone "B" over a shorter period of time, and, especially to establish more precisely its effect on thyroid I^{131} concentration, an acute experiment was devised. This was divided into three sections. Female rats weighing between 130 and 220 g were used for each determination, the weights being relatively constant in each section; all injections were by the subcutaneous route. The first section of the acute experiment was planned in such a way that Amphenone "B" was given both before and after the injection of radioiodine in order that a maximum effect on thyroid I^{131} concentration might be produced. TSH§ was given to compare its action with that of Amphenone "B". The short-term effects of the compound on adrenal cholesterol concentration were also observed in this section. Seven groups of 4 rats each were used. Three groups were given Amphenone "B" (10 mg in saline at 0 hours, 5 mg in saline at 4 hours, 10 mg in sesame oil at 7 hours, and 10 mg in saline at 18 hours; a total of approximately 20 mg per 100 g of body weight), 50 μc of I^{131} at 6 hours, and killed at 24 hours. Two groups received I^{131} alone and served as controls. In all 5 groups the measurements consisted of thyroid weight and I^{131} concentration, adrenal weights and cholesterol concentration and thymus weight. A sixth group received I^{131} at 6 hours, 12.5 mg of TSH at 19 hours and were sacrificed at 24 hours. A seventh group was given Amphenone "B", I^{131}, and TSH at the times mentioned above. Thyroid weights and I^{131} concentrations alone were determined in the last 2 groups. In the second section of the experiment, radioiodine was given 16 to 17 hours before the administration of Amphenone "B" or TSH. It was hoped that this dosage schedule would demonstrate whether or not Amphenone "B" produced an increased release of thyroid I^{131}. Eight animals were used in each group. The dosage schedule was as follows: 50 μc of I^{131} was given at 0 hours, 10 mg of Amphenone "B" in saline at 16 hours, 12.5 mg TSH at 17 hours and the rats were killed at 22 hours. One group was given all the above, one group was given I^{131} and TSH, one group was given I^{131} and Amphenone "B", and a fourth, control group, was given I^{131} alone. Thyroid weight and I^{131} concentrations were determined as above. In a third experiment, 3 groups of 8 animals were used. Here, the dosage schedule was arranged so that the possible effects of Amphenone "B" on iodine uptake by the thyroid might be demonstrated. The animals were given Amphenone "B" (10 mg in saline at 0 hours, 10 mg in saline, and 10 mg in oil at 6 hours, and 10 mg in saline at 16 hours) 12.5 mg of TSH at 16 hours, 50 μc of I^{131} at 16 hours and were killed at 22 hours. One group received all the above, one group received TSH and I^{131}, and one group, the control, I^{131} alone. Thyroid size and radioiodine concentration were determined.

Results. In the chronic experiment (Fig.

§ Kindly supplied by Dr. Harley Cluxton, Armour and Co., Chicago, Ill.

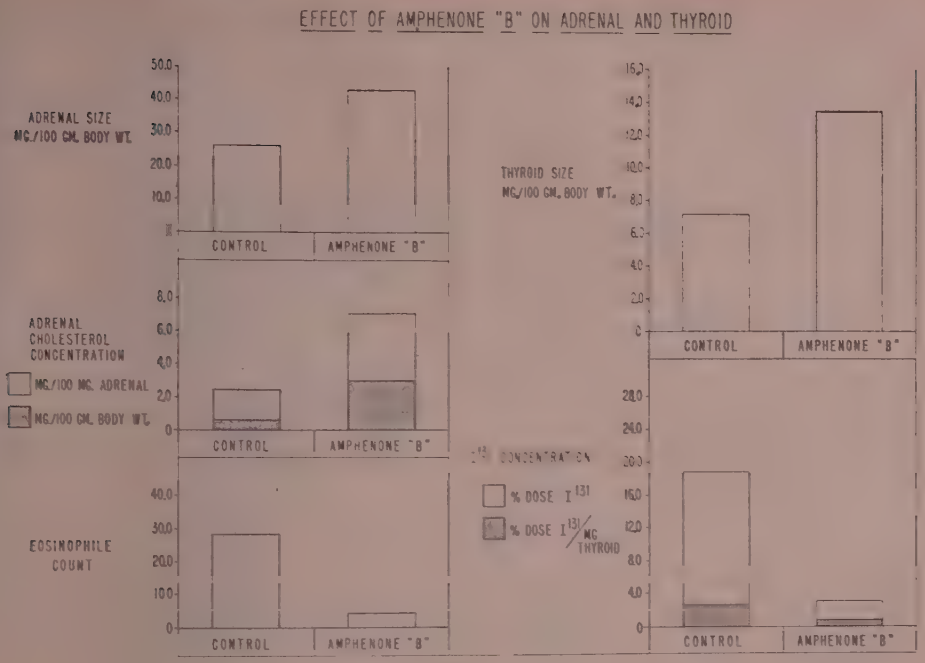

FIG. 1.

1) the adrenal size increased approximately 60% with the administration of Amphenone "B". Moreover, the adrenal cholesterol concentration increased about 200%. At the same time the number of circulating eosinophiles decreased to 12% of that of the controls. The thyroids increased in size from an average of 7.4 mg per 100 g body weight in the controls to 13.6 mg in the treated animals, while the I^{131} content in the Amphenone "B" rats fell to 16% of that of the controls. All animals were weighed before, during and at the end of the experiment and both the controls and the Amphenone "B" treated animals exhibited a constant weight gain.

Thymic weight decreased from 300 $\pm$ 11.8 mg per 100 g of body weight (mean $\pm$ standard error) in the controls to 252 $\pm$ 25.3 mg in the Amphenone "B" treated animals. The testes decreased in size from 773 $\pm$ 48.6 mg per 100 g body weight to 624 $\pm$ 41.2 mg. The figures for the testes are statistically significant, the P value being less than 0.05. Those for the thymi are significant only at the 10% level as P is greater than 0.05.

The acute effects of Amphenone "B" on the thyroid are shown in Fig. 2. It will be seen that in this experiment, with the administration of Amphenone "B" on four occasions within a 24-hour interval, the effect on I^{131} concentration is even more striking than in the chronic group. The concentration of I^{131} in the thyroid was less than in the controls whether Amphenone "B" was given before or after the administration of radioiodine. In addition, TSH when given alone at the time intervals indicated, produced a decrease in I^{131} concentration whether expressed as total content or as concentration per mg of thyroid. The combination of Amphenone "B" and TSH resulted in no significant difference in I^{131} concentration from that seen with Amphenone "B" alone. Where both substances were given before the administration of I^{131}, although the total radioiodine in the hypertrophied gland was equal to that of the control the concentration in the gland was low. In the acute experiment there was no significant change in adrenal size or adrenal cholesterol concentration. There was no change in thymic size of

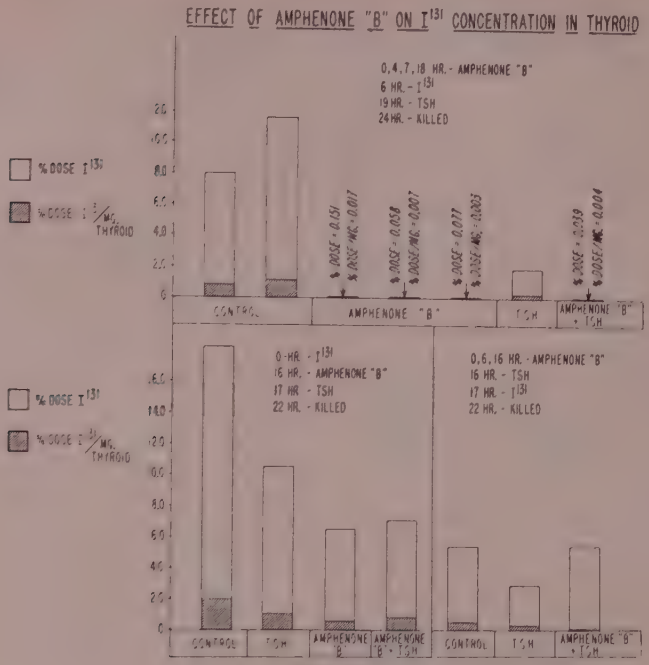

FIG. 2.

Amphenone "B" treated animals within this short period.

Discussion. Adrenal. The increase in adrenal size observed in our experiments confirms that reported originally by Hertz *et al.* (2). The fact that they did not find this effect in hypophysectomized animals and that the response was diminished or absent in animals given cortisone is good evidence that Amphenone "B" produces an increased secretion of adrenocorticotropin. Not only an absolute but a marked relative increase in adrenal cholesterol was noted in all Amphenone "B" treated animals. Sayers and coworkers(8) have shown that although single doses of ACTH result in depletion of adrenal cholesterol, more prolonged administration of this hormone may produce an elevation in adrenal cholesterol concentration, providing determinations are made 16 hours or more following the last injection of ACTH. This evidence suggests that Amphenone "B" causes adrenal enlargement by increasing the secretion of ACTH. A marked fall in circulating eosinophiles was noted in animals treated with Amphenone "B" over an 11-day period. This finding indicates that despite accumulation of large amounts of cholesterol, the hypertrophic adrenals are capable of secreting sufficient quantities of steroid to cause such a response. Hence, Amphenone "B" does not have an effect on the adrenals analogous to the effect of thiouracil on the thyroid since the adrenals are able to produce an increased amount of steroid. Were this a "thiouracil-like" effect of such a degree as to produce the marked hypertrophy and increase in cholesterol concentration seen in these experiments, one would expect to find almost complete blocking of adrenal hormone secretion.

Thyroid. Despite an almost 2-fold increase in thyroid size observed in Amphenone "B" treated animals, there was a marked reduction in the total amount of I^{131} and hence an even more marked decrease in I^{131} concentration. The latter effect was observed in both chronic and acute experiments and was most pronounced in the acute, where Amphenone "B" was given at shorter intervals over a relatively short period of time. It is known that TSH

promotes both an increased uptake of I^{131} by the thyroid and an increase in its secretion into the blood as thyroxine. With the dosage schedules of all sections of our acute experiment the latter phase apparently predominated, as in all instances I^{131} concentration was significantly lower following the administration of TSH than in the control. Amphenone "B" produced an effect on thyroid I^{131} concentration similar to that of TSH. The acute experiments were originally designed to show whether the drug exerts its effect primarily on the uptake of I^{131} or on its release. Since, contrary to expectations, thyroid I^{131} concentration was low whether Amphenone "B" was given before or after a tracer dose of I^{131} it may be that both factors are involved or, more likely, the action of the drugs administered was exerted over such a length of time as to obscure interpretation as to mode or site of action. Studies are in progress to clarify this point. These observations indicate therefore, that the action of Amphenone "B" on the thyroid may be through direct and immediate stimulation of production of TSH by the pituitary and/or due to a blocking effect such as that seen with thiouracil.

Testes. In the chronic experiment, Amphenone "B" produced a decrease in the size of the testes, although no consistent diminution in size or number of any one cell type was observed. Inasmuch as the compound is known to have a progestational action, it may be postulated that its presence caused a diminished secretion of FSH by the pituitary leading to testicular atrophy. Similarly, the presence of excessive amounts of adrenal corticoids following Amphenone "B" administration may also have caused a reduction in FSH secretion.

Summary. (1) Enlargement of rat adrenal and thyroid glands following the administration of Amphenone "B", observed by Hertz, Allen, and Tullner, was confirmed. (2) The adrenal enlargement was associated with an increase in adrenal cholesterol concentration. At the same time, the adrenals remained capable of secreting sufficient steroid to produce a marked fall in circulating eosinophiles. Amphenone "B" may exert its adrenomegalic effect by direct stimulation of the adenohypophysis to produce ACTH rather than indirectly as seen, for example, in thiouracil-induced goiter. (3) In addition to thyroid enlargement, a marked decrease in I^{131} concentration in the gland was observed. This could be due to a thiouracil-type of action, although the effects noted within the time relationships of our experiments were similar to those produced by TSH and hence possibly the result of increased secretion of radioiodine rather than a decreased uptake. (4) Decrease in size of the testes was observed in animals given Amphenone "B". It is postulated that this may be due to a diminished production of FSH brought about either by elevated blood levels of Amphenone "B" itself or by an increase in adrenal corticoids as a result of adrenal stimulation.

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Preparation of Specific Complement-Fixing Antigens from the Three Prototypes of Poliomyelitis Viruses.* (19268)

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Specific complement fixing (CF) antigens have been prepared from nerve tissues infected with Lansing poliomyelitis virus by technics involving acetone desiccation(1), methanol precipitation(2), and ultracentrifugation(3). Lansing poliomyelitis virus was adapted to new-born mice, and from this adapted virus, a CF antigen was prepared which appeared to show some antigenic relationship to Brunhilde type antibody contained in monkey and in human antiserum(1). While extensive studies involving neutralization and vaccination-challenge technics indicate that Lansing, Brunhilde and Leon viruses are antigenically distinct(4,5), one might speculate either that the Lansing virus may have been altered by such an adaptation or that the method used in preparing such CF antigens did not yield a specific antigen. Since the technic employed has repeatedly yielded specific CF antigens with other neurotropic viruses (6), the latter supposition might thereby be vitiated. It should be pointed out that the Brunhilde serums used above were not tested for CF properties with Brunhilde antigen. More recently a specific CF antigen was prepared from Lansing infected cotton rat brains by a technic involving acetone desiccation as referred to above(7).

Prior to the use of adjuvants in immunization procedures, it was very difficult to demonstrate CF antibodies in immunized monkeys. It was felt at that time that perhaps monkeys did not produce such antibodies. By mixing the virus inoculum with depot-producing agents, monkeys thus immunized rapidly responded with high titer neutralizing antiserum and rapidly developed cerebral immunity to challenge with homologous virus(8-10). Since specific CF antigens were consistently produced from Lansing infected cotton rat (LCR) tissues by the ultracentrifugation technic(3), it was felt that nerve tissues infected with the Brunhilde and the Leon types of poliomyelitis virus should be processed in the same manner for specific CF antigens.

Methods. Antigens. The method employed has been previously described(3) and will be briefly outlined as follows: Spinal cords of *Macacus mulatta* monkeys or brains of cotton rats *(Sigmodon hispidus)* collected early in the course of symptoms of poliomyelitis were emulsified in cold 0.15 M NaCl by a Waring blendor (6 minutes). The final dilution of cord material was a 20% suspension by weight. The emulsions were clarified at 5000 rpm for 1 hour and 18,000 rpm for 10 minutes. The clear supernatant fluid was mixed with ⅓ volume di-ethyl ether and stored at 4°C for one hour. The mixture was centrifuged at 5000 rpm for 30 minutes and the aqueous portion thereof was stored at –20°C overnight. The thawed and clarified suspension was then centrifuged in the Spinco preparative ultracentrifuge at 144,000 x G for 2 hours. The sedimented pellet was resuspended in 1/10 volume M/5 phosphate buffer (pH 7.2) stored overnight at 4°C, and after centrifugation at 5000 rpm for 30 minutes, the clear supernate was tested as antigen. CF antigens were thus prepared from cotton rat tissues infected with Lansing virus; and from monkey tissues infected with Brunhilde, Wisconsin, and Leon viruses.†

Antiserum. The 3 prototypes of poliomyelitis virus (Lansing, Leon and Brunhilde) were individually mixed with a depot producing adjuvant,‡ and inoculated at weekly intervals into the gastrocnemius muscles of *M. mulatta* monkeys. The monkeys were each bled at

* Aided by a grant from the National Foundation for Infantile Paralysis.

† Viruses were kindly supplied as follows: Brunhilde and Leon by Dr. David Bodian; Wisconsin by Dr. H. K. Faber.

‡ "Pendil" manufactured by Endo Products, Richmond Hill, N. Y., contains oxycholesterol, peanut oil, and beeswax.

TABLE I. Specificity of Complement-Fixing Antigens Prepared by Ultracentrifugation of the Virus.

Monkey serum	Antigens					
	LCR*	Brunhilde	Wisconsin	Leon	Rabies	SLE
Lansing	4+ 1:40	—	—	—	—	—
Brunhilde	—	4+ 1:5	4+ 1:5	—	—	—
Leon	—	—	—	4+ 1:5↑	—	—
Frederick	—	4+ 1:5	4+ 1:10	—		

* LCR—Lansing virus in cotton rat tissues. Rabies—in cotton rat tissues. SLE—St. Louis encephalitis virus in cotton rat tissues.

weekly intervals and the clear serum was stored at –20°C until used. Antiserum specimens were also kindly supplied by Drs. Jonas Salk (Brunhilde and Leon), C. F. Pait (Leon), and H. A. Howe (Frederick). The serums from Dr. Salk were prepared by the technic employing a depot-producing adjuvant (Arlacel A and Bayol F) as an antibody enhancing agent(9).

C.F. test. The serums were inactivated (1 in 5 dilution) at 61°C for 20 minutes. Two-tenths cc each of serum, antigen, and complement (2 units) were mixed and incubated at 4°C for approximately 18 hours. The amboceptor and 2% sheep red blood cells (0.2 cc each) were added and the mixtures were stored at 37°C for 30 minutes at which time the results were recorded. Serum was titrated in a constant dilution of antigen and antigen was titrated in a constant dilution of serum. All CF tests were set up at least 3 times. Serum, antigen, and complement controls accompanied each test. CF antigens similarly prepared from cotton rat tissues infected with rabies and with St. Louis encephalitis (SLE) viruses served as further controls on the specificity of the antigens.

Results. As indicated in Table I, specific complement fixation was obtained with Lansing antigen and antiserum. Leon antiserum fixed complement with Leon antigen, and Brunhilde antiserum with Brunhilde antigen. Antigen prepared from Wisconsin infected monkey tissue fixed complement with Brunhilde and with Frederick antiserum. The antigens prepared from monkey tissues were not potent beyond 1:1 dilution and often had to be used undiluted. The LCR antigen could often be used at 1:2 and 1:4 dilution. The latter antigen retained its specificity when stored for at least 80 days at –20°C. Not all Brunhilde antigens prepared were suitable as CF antigens; whereas, those prepared from Wisconsin virus were more consistently useful. The water clear antigens were neither anticomplementary nor procomplementary. The average yield of antigen per gram of tissue was 0.5 cc.

When 4 monkeys were immunized with Lansing virus-adjuvant mixtures, they responded within two weeks with specific CF antibodies. Two monkeys without adjuvant very gradually developed detectable low titer CF antibodies after 12 weekly inoculations. When adjuvant-virus was inoculated into the latter monkeys, they responded within 1 week with CF antibody titers of 1:40 and 1:80. The CF antibody levels of all virus-adjuvant inoculated monkeys remained elevated for at least six months without further exposure to antigenic stimulus.

Two monkeys which were inoculated with Brunhilde-adjuvant and two with Leon-adjuvant mixtures did not produce detectable CF antibodies even after nine inoculations. The antigens used to test these serums did fix complement specifically with the Leon and the Brunhilde antiserums that were supplied by Dr. Salk.

Discussion. The specific serological differentiation exhibited by the three prototype viruses is in agreement with the results attained by others through serum neutralization and vaccination-challenge technics(4,5). The close relationship demonstrated for Brunhilde, Wisconsin and Frederick viruses is in accord with the above findings(11). The CF serum titers of the non-Lansing tests (Brunhilde & Leon), while relatively low, were significant. It must be emphasized that the CF antigens prepared from the latter 2 viruses were difficult to attain. That not every at-

tempt was successful might be attributed to the initial low titer of virus in the tissues used or that the virus may have been dissipated while being processed. Specific LCR CF antigen is consistently produced by the technic described. While it was difficult to process consistently satisfactory CF antigens from Brunhilde infected cords, the comparative success with Wisconsin virus has caused us to concentrate our efforts on the latter virus. It is possible that another virus might be superior even to the latter.

We have prepared specific Lansing CF antigens by the acetone-ether technic previously described(6,7) from infected brains of mature cotton rats. Thus far, similar attempts with Leon and Brunhilde-infected monkey tissues have not yielded CF antigens when tested with suitable antiserums.

The adjuvant "Pendil" as used by us did not enhance the CF antibody response of monkeys to Brunhilde and Leon viruses as did the Arlacel A and Bayol F of Salk *et al.*(9). Why "Pendil" worked with LCR and not with Brunhilde and Leon antigens is as yet beyond explanation. The advantage of the depot-producing agent with LCR is apparent and explains the CF difficulties in past experiences with antiserums prepared in monkeys. This is a good example of how suitable CF antigens might be discarded because of unsuitable antiserum, the latter presumably being produced by sound technics.

We have demonstrated specific CF with the three prototype viruses of poliomyelitis. Whether this will have a practical application will depend on future development with the virus and on the serological pattern demonstrated in human serums. Thus far the CF antigens processed from Leon and Brunhilde-type infected tissues were neither easily prepared nor potent when finally attained. We still need a better source of virus than that used for this study.

Summary. Specific complement-fixing antigens were prepared from Lansing-, Brunhilde-, and Leon-infected tissues by sedimentation of the viruses in the high speed centrifuge and using the resuspended sediment as antigen. Specific complement fixing antiserums were produced more rapidly in Lansing immunized monkeys when the virus inoculum was incorporated into a depot-producing adjuvant. It appears that the adjuvant treatment as recommended by Salk is needed for production of Brunhilde and Leon complement fixing antiserum.

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Acute Disseminated Encephalomyelitis Produced in Mice by Brain Proteolipide (Folch-Lees).* (19269)

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The isolation and identification of the encephalitogenic agent present in brain tissue has been sought since experimental disseminated (allergic or isoallergic) encephalomyelitis was first definitely produced(1). Various fractions deriving from extracts of cerebral tissue by lipid or other solvents—substances considered by some investigators to be leci-

thins, cephalins, cerebrosides, phosphatides, or protein, and by others, to be lecithin- or lipid- or protein-free residues—were reported now and again as encephalitogenic. Monkeys, rabbits and guinea pigs chiefly were the experimental animals but few of them were studied, and consistent results were not always had, and positive findings were, therefore, generally reported with caution(2). Recently, Ferraro and Roizin(3) investigated the effects of a calcium-acetate soluble compound obtained from benzene-ether extracted rabbit or sheep brain prepared by themselves or by Habel, Bell and Wright(4,5). This compound was designated(3) as being predominantly protein and induced either neurological symptoms of encephalomyelitis in 12 of 25 guinea pigs, or in 5 others showing no such signs, characteristic lesions in the CNS. Ferraro and Roizin concluded that while this protein calcium acetate fraction was more actively encephalitogenic than others heretofore reported, it was still not as potent as whole brain; they suggested, consequently, that either more than one substance in brain tissue might be active or the lipid fractions hitherto described as producing the affection might have been contaminated by a more potent protein fraction. Finally, Vogel(6) showed by both histologic and cytochemical means that a lipolytic enzyme was found in affected guinea pigs in the cytoplasm of a large proportion of the reactive histiocytes in the granulomatous tissue at the site of injection, and in the inflamed regional lymph nodes. He suggested, therefore, that a lipolytic enzyme may be a factor in the pathogenesis of the experimental affection and its accompanying demyelinization. No uniform opinion exists, therefore, on the identity or nature of the encephalitogenic agent in brain tissue.

The subject of the identity of the encephalitogenic agent is being studied in this laboratory in Swiss-W mice(7), especially those of certain stocks, such as the H-line, which have been shown to be highly susceptible(7-9). Several attempts have been made during the past 3 years to secure an active material, chiefly from mouse brain, and in all instances, with the aid of the Freund type of adjuvant(7). Thus an acetone-ether "lipid" fraction already described(7) failed, as did also the residue, or non-lipid moiety. Negative results were also obtained with pyridine-precipitated or extracted brain tissue, also with dialyzed material after 2 repeated extractions by pyridine, or with dialysates obtained from brain homogenates carried out against distilled water through a Visking cellulose membrane (9). In addition, a fraction insoluble in chloroform, also in chloroform methyl alcohol, and dialysates of such extracted cerebral tissue were used, but without success. Finally, chemical substances, such as folic acid, galactose, acetylcholine, glutamic acid, and a protein-lipocarbohydrate complex derived from Shigella antigen, were likewise without noteworthy results. In the face of all these failures, the success achieved with brain "proteolipide A and B", a new type of tissue lipoproteins recently discovered by Folch and Lees(10) was striking, and is the subject of the present paper.

Methods and materials. The method of adding Freund-type adjuvant to materials to be injected into H-line Swiss-W mice; the nosography of the affection induced in them; the dosage; route of inoculation; period of observation, and age of animals were already fully described(6,8,9). It should be mentioned again, however, that the standard course of treatment comprised 6 injections given at weekly intervals or until neurological signs appeared when further inoculations were discontinued. The materials used were first, a sample of proteolipide A secured through the kindness of Dr. J. Folch-Pi, a preparation deriving from cattle cerebral white matter. The second material was made in this laboratory from whole brains of 150 uninoculated adult stock mice, equivalent to 60 g of wet tissue. The preparation followed closely the method of Folch and Lees(10) up to the steps of processing the fluff formed at the interphase between water and chloroform. The process was con-

* Thanks are due Dr. G. Middlebrook for a supply of tubercle bacilli; to Ann Behrer for technical assistance, and to Dr. J. Folch-Pi, McLean Hospital, Waverly, Mass., for a sample of cattle-brain proteolipide A and for his generous cooperation.

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tinued to the point where the fluff was collected on filter paper, thus containing both proteolipide A and B. These proteolipides as defined by Folch and Lees are a group of lipoproteins consisting of lipid and protein components; they are soluble in chloroform-methanol-water mixtures but insoluble in water, thereby differing from the known lipoproteins, and thus forming a new distinct group. The A form is a mixture of proteolipides and of free lipids; the B, an isomorphic crystalline product probably of one or more proteolipides with cerebrosides. Neither A nor B types are regarded as pure substances(10). The A and B mixture was used in the present tests. Before use, however, the mixture was aerated to rid it of chloroform, of which an amount sufficient to be toxic for inoculated mice was present.

Experimental. For an experiment of orientation the proteolipide A fraction of cattle brain prepared by Folch and Lees was first studied. It was added to adjuvant in the following amounts: 0.5 g proteolipide A; 125 mg autoclaved tubercle bacilli; 10 mg merthiolate; 25 ml saline solution, and 25 ml heavy mineral oil, homogenized in a Waring Blendor. Thus the proteolipide was diluted about 1:100, and 20 mice were inoculated with it and of them only 12 received the full course of 6 weekly injections, and owing to lack of material, 8 were given only 4 injections. Of the 20 animals one showed paralysis of one posterior extremity after 3 injections; the others were apparently free from symptoms. Histopathological examination revealed that the paralyzed mouse, 4 others of the 12 receiving full dosage, and 2 of 8 after only 4 injections had lesions in the CNS characteristic of the experimental encephalomyelitis. Attention has already been called to the fact that in this experimental affection, mice can be symptom-free yet show a varying degree of specific lesions in the CNS(8). It appeared then that the proteolipide A of cattle brain was encephalitogenic in mice. Its activity was such that with 10^{-2} dilution, only 7 of 20 mice showed either signs of characteristic reaction or its specific pathologic picture or both. Reference has already been made to the fact that in mice heterologous tissue is less potent than homologous. The second experiment was therefore performed with proteolipide A and B prepared in this laboratory deriving from normal mouse brain as described in a foregoing paragraph (Methods and Materials).

For animal inoculation, the undiluted proteolipide from 150 mouse brains was added to 100 ml of the Freund-type adjuvant and the dosage used for mice was the same as that of the cattle-brain proteolipide A. Four sets of animals were inoculated, of which 3 were controls. The first control consisted of 20 mice receiving untreated mouse brain plus adjuvant in similar proportions; this constituted a "positive" control to check the reactivity of the stock mice and the encephalitogenic power of their brain tissue. The second and third controls comprised 40 mice which received adjuvant and the residue of the tissue after the proteolipide had been extracted; the second series, 6 times the concentration of tissue as ordinarily used and as represented in the third set. The object here was to find out whether all of the encephalitogenic agent was removed from the tissue residue. The final series was the test itself with the proteolipide-adjuvant mixture, in which 28 mice were injected with it. Table I summarizes this experiment. Another series of 20 mice is not shown in the table. In this the animals received a single injection of proteolipide before its aeration to remove chloroform; 15 of the animals succumbed to chloroform poisoning within a few days; the remainder were unaffected. It is clear from the results that the mouse-brain proteolipides are actively encephalitogenic in mice, apparently to a greater degree than is whole mouse brain. Moreover, it would appear that most, if not all of the active agent had been extracted from brain tissue, for only 2 of 35 mice given the extracted residue developed experimental encephalomyelitis, the reactors having received the highly concentrated material.

Discussion and summary. Several conventional fractions of brain tissues and other chemical substances failed to produce encephalomyelitis in highly susceptible mice until a new group of lipoproteins, the proteolipides recently discovered by Folch and Lees were tried. Then the results were arresting: for not only were mouse-brain proteolipides as

TABLE I. Effect of Proteolipide A and B (PAB) in Mice.

Material inj. + adjuvant	No. of mice inj.	No. with signs	No. with CNS lesions; symptom-free	Total No. positive	Avg days to 1st sign
Normal mouse brain	20	19	0	19	25
Residue after PAB extracted	20	2	0	2	15
Residue diluted 1:6	15	0	0	0	—
PAB	28	24	3	27	14.6

actively encephalitogenic as whole brain, perhaps even more so, but all of the active agent could be practically extracted; the slight degree of activity shown by the residue (Table I) could have been ascribed to its possible contamination with proteolipides.

Two striking features of this new encephalitogenic material are worthy of note. The first is that since proteolipides are absent from unmyelinized brain, it is apparent that they are components of myelin (Folch and Lees) (10). It is well known that demyelinization is characteristic of the pathologic picture of disseminated encephalomyelitis. The second is the finding of Waksman and Morrison(11) who employing proteolipide A and B prepared by Folch and Lees showed weakly positive skin reactions with it in rabbits having experimental encephalomyelitis induced by injection of homologous spinal-cord tissue. The quantitative difference in the results obtained after inoculation of mice with Folch and Lees' own preparation of proteolipide A and the mouse-brain A and B substance prepared in this laboratory might be considered as an insufficiency of active agent in the A fraction by itself, or as a demonstration of the greater susceptibility of animals to homologous brain tissue. In view of the fact that the proteolipides are compounds constituted by lipid and protein moieties (Folch and Lees) further study is planned on possible purification of the active agent. Finally, with respect to the human demyelinating encephalitides to which the experimental affection has certain resemblance the problems are: What is the relationship of the proteolipide to their causation? Can proteolipide function as a test for diagnosis, perhaps in the way antigens are employed in allergic disorders?

Conclusion. Proteolipide A and B of Folch and Lees, a new group of lipoproteins, are capable of bringing about in mice acute disseminated encephalomyelitis indistinguishable from the affection induced by inoculation of whole brain tissue. The substance contains practically all of the encephalitogenic agent present in brain.

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Local Hypersensitiveness of the Rabbit Stomach.* (19270)

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A local state of hypersensitiveness restricted to the organ which was the site of the primary injection, was obtained in the eye with exogenous antigens by Seegal and Seegal(1); in the eye, cerebrum and lung with exogenous and autogenous antigens by Jahiel *et al.*(2-5) and in the cerebrum with exogenous antigens by Davidoff *et al.*(6). These experimental studies were performed chiefly in rabbits, and following the intravenous shock injection lesions were noted in the organ which had received the primary sensitization.

The present series of experiments were made to determine whether the rabbit's stomach is capable of becoming actively and locally hypersensitive and to study the nature of the lesions obtained.

Material and methods. Horse serum was used as an antigen. Fifty rabbits were sensitized locally, after ether anesthesia and laparotomy, with .05-.8 cc horse serum injected with a 27-gauge needle subserosally in the stomach wall; after a free interval varying from 9 days to 9 weeks, they were tested with 1 to 5 cc of the same antigen injected intravenously. Twenty-one control rabbits received only the sensitizing injection. Thirteen other controls received only the intravenous injection. The rabbits were killed or died at various intervals after injection. Their stomachs were compared with those of a series of 70 untreated animals. All animals came from the same source for each series of experiments and their controls. They weighed approximately 3 lb when purchased and remained in the laboratory for at least one week before use. During that period and postoperatively they were fed uniformly.

Results. Normal untreated rabbits revealed in our laboratory 8% of localized spontaneous gastric lesions or ulcerations. Twenty-six of the 50 experimental animals had gastric lesions or ulcerations representing an incidence of 52%. Four of the 34 controls had gastric lesions representing an incidence of 12%. Thus the incidence of lesions in the group of rabbits locally sensitized and tested afterwards by intravenous injection was significantly greater than those in either series of controls, indicating that a phenomenon of local hypersensitiveness can be obtained in the rabbit's stomach.

The lesions varied in intensity in the experimental animals. The most discrete ones consisted of a localized area of mucosal and submucosal edema with moderate infiltration with lymphocytes, plasma cells, and a few eosinophilic cells. Less discrete ones showed localized hemorrhagic foci in the lowermost part of the mucosa. When the process was more extensive, the hemorrhagic and exudative lesions extended throughout the mucosa with minute areas of necrosis and erosions. In the most extensive lesions there was an actively bleeding ulceration extending to the muscularis mucosae with inflammatory reactions in the underlying submucosa but no erosion through the muscularis mucosa. In a few cases these varied lesions were seen in the same animal.

The factors common to these lesions were: (1) the predominance of the vascular features, namely, edema, hemorrhage, endothelial reaction, perivascular infiltration particularly around minute vessels; no lesions in the vessel wall were encountered however; (2) the constant involvement of the lowermost part of the mucosa. The lesions appeared within 2 hours after the shock injection. In the animals killed within the first 24 hours active edema and hemorrhage were present and necrosis and cellular infiltration were already observed. In the animals killed after 24 hours, the edema was much decreased or absent; there were no fresh hemorrhage, and necrotic areas infiltrated with polymorphonuclears were the most noteworthy features.

* This investigation was supported (in part) by a research grant from the National Institutes of Health, Public Health Service.

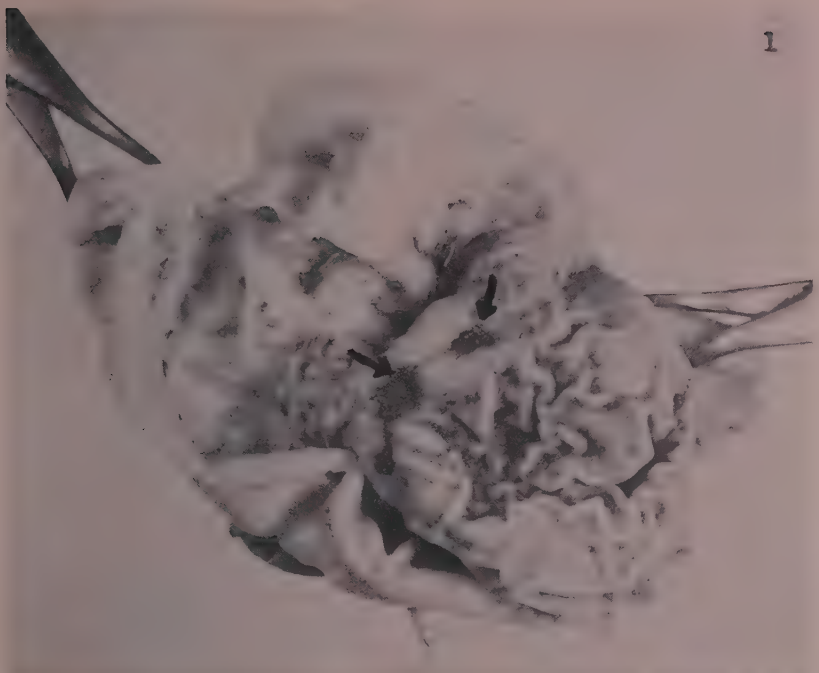

FIG. 1. Rabbit 27. Sensitized after laparotomy, with .2 cc horse serum. Shocked 21 days later with 2 cc horse serum intrav. Killed 3½ hr after injection. Hemorrhagic ulcerations in supracardiac region.

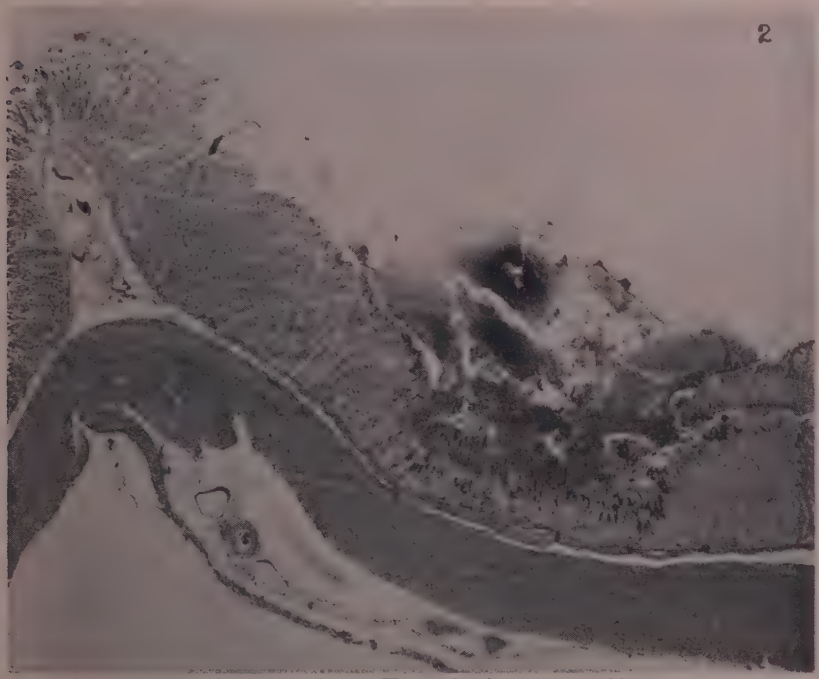

FIG. 2. Rabbit 27. Hemorrhagic crater, × 30.

The location and number of lesions varied. They were found near the cardia, in the lesser curvature, at the pyloro duodenal junction, or in the anterior and posterior wall near the cardia. They were not found along the greater curvature. The lesions were not at the exact site of the sensitizing injection but were within a wider area of the stomach mucosa. We interpret this fact as due to variations in diffusion of antigen at the time of the sensitizing injection. The lesions occurred as either single or 2 or 3 separate erosions or ulcerations with areas of edema in between them. The incidence and severity of the lesions were not significantly affected by variations in the doses or the time interval between the two injections within the range used in these experiments.

No apparent lesions were found in other organs of the experimental animal. When spontaneous lesions were observed or when lesions were seen in the control animals, they were similar to the ones observed in the experimental animals, namely, edematous, hemorrhagic or ulcerative lesions (except for an occasional infection).

Comment. Anaphylactic reactions of the Arthus type were described in the stomach of generally sensitized animals by various observers (Shapiro and Ivy(7), Alessio(8), de Gara and Angevine(9).) Cytotoxins were used by Bolton(10), and Myagawa(11). Anaphylactic reactions in the passively sensitized stomachs of dogs and monkeys were produced following intravenous injection of antigen by Walzer(12) and Friesen(13). To our knowledge, no attempt was made until now to obtain a local active hypersensitiveness of the stomach. This organ responds to the phenomenon of local organ hypersensitiveness with lesions variable in number and intensity. Organs other than the stomach have not shown lesions following primary sensitization of this organ.

Compared to peptic ulcer in man, the lesions obtained represent in most of the cases, erosions rather than ulcers according to Ivy's classification(14). They may resemble lesions seen early in the disease. Bernard described recently(15) scattered "intramural" lesions in the stomachs of patients with ulcers. These lesions were detected only by serial sections of specimens of gastrectomy; they were sometimes located at a certain distance from the ulcer crater; they did not reach the surface of the mucosa, hence they were not visible in the gross specimen. Although such a study, by serial histological sections, was not performed on our experimental specimens, some of our slides showed lesions of the lowermost part of the mucosa resembling the intramural aspects observed in human stomach by Bernard.

Summary. (1) A phenomenon of local organ hypersensitiveness, using an exogenous antigen (horse serum) was produced in the rabbit stomach. (2) The stomach wall, at the time of laparotomy was the site of primary sensitization. After an interval of several days to several weeks, the shock injection was given intravenously. (3) The lesions obtained were single or multiple. In a number of animals actively bleeding lesions with crater formation surrounded by edema were produced. (4) The stomach was the sole organ grossly involved.

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Blood and Liver Glutathione During Protein Deprivation.* (19271)

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The concentrations of both liver xanthine oxidase(1) and glutathione(2) are grossly reduced during protein deprivation. The purpose of this study was: (1) to compare the relative patterns by which these two constituents are lost from the liver during protein depletion, and (2) to see if blood glutathione reflected the liver changes so that the former might possibly serve as a criterion of protein or amino acid nutrition. Xanthine oxidase provided an index of the degree to which each liver was affected by a protein-free diet, and was therefore used as a reference point for comparative purposes.

Experimental. Adult male Sprague-Dawley rats previously maintained on Purina chow were placed on a protein-free diet for 1 to 22 days. This diet was identical with the 21% casein diet previously published(3) except that glucose replaced the casein. Groups of rats were sacrificed by decapitation at intervals and analyzed for liver xanthine oxidase (4), liver and blood glutathione. One group of rats was fed the protein-free diet to which 1% cystine was added. Reduced glutathione was determined in a sulfosalicylic acid filtrate of liver homogenate and blood by the iodometric titration of Woodward and Fry(5) after removing ascorbic acid(2). Total glutathione was determined similarly after reduction of the filtrate by the addition of Zn dust (5). Added glutathione was recovered from

TABLE I. Effect of Protein-Free Diet on Rat Blood and Liver Glutathione.

Diet			Body wt, g			Liver		Blood		
Type	Days	No. of rats	Start	End	Wt. of 100 g rat	Xanthine oxidase, Cmm O_2/20† min, 283 mg	Reduced glutathione, mg/100 g	Total glutathione, mg/100 ml	Reduced glutathione,* mg/100 ml	Hematocrit, %
Chow	—	9	—	278	3.5	28 (24-34)	240 (167-348)	32 (20-43)	24 (18-33)	39
	1	8	237	229	3.7	20 (13-30)	134 (95-142)	29 (21-42)	27 (19-36)	39
	2	4	265	260	3.3	14 (9-19)	96 (77-109)	32 (22-40)	27 (21-33)	43
	5	4	255	238	3.6	8 (0-16)	102 (99-109)	39 (28-45)	—	41
Protein-free	6	4	284	266	3.6	5 (1-10)	84 (72-99)	45 (20-56)	—	41
	8	4	268	234	3.4	9 (0-23)	117 (86-150)	—	26 (23-30)	38
	16	4	270	215	2.7	2 (0-10)	111 (93-136)	—	33 (28-41)	33
	22	4	274	212	2.8	1 (0-2)	86 (76-95)	—	48 (40-65)	35
Protein-free + 1% cystine	6	4	298	272	3.4	9 (8-10)	442 (381-514)	—	34 (26-37)	44

Values in parentheses give the range of the results.

* The values for reduced blood glutathione are not directly comparable with the total blood glutathione since both determinations were not run simultaneously on all blood samples.

† Net oxygen consumption with hypoxanthine substrate in Cmm O_2 per 20 min per Warburg flask containing 283 mg fresh liver.

* This work was supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

blood and liver by this procedure with an average error of +3 to 4% (range 100-106%).

The results are shown in Table I. Liver glutathione was reduced to nearly one-half its normal value by feeding a protein-free diet for 1 day, and it remained constant at about 40% of the starting value thereafter. Blood glutathione values were not decreased by protein deprivation; some increase was noted, first in the oxidized and then in the reduced glutathione, as the diet period progressed. This increase was not the passive result of an altered hematocrit, and may have been due to glutathione itself or to some unidentified interfering substance. Like the glutathione in normal blood(6) the reacting substance was localized within the red cell, since the plasma of rats fed a protein-free diet for 23 days was devoid of glutathione. Blood glutathione was not affected by protein depletion in such a way that its determination would be of value in assessing protein nutrition.

Feeding a protein-free diet containing 1% cystine did not prevent the loss of liver xanthine oxidase that occurred during protein deprivation, but the added cystine increased the liver glutathione markedly and the blood glutathione slightly. In a similar study Leaf and Neuberger(2) found that both cystine and methionine added to a protein-free diet restored the true liver glutathione to normal or slightly elevated values. The very high levels of liver glutathione, which they also obtained with this iodometric titration, were apparently due to a non-thiol interfering substance.

Protein depletion decreases liver xanthine oxidase and glutathione markedly, but has no large effect on these constituents in kidney, lung, muscle, or spleen(7,2). Xanthine oxidase in blood(8) and intestine(4) is also affected by a low protein diet, while glutathione is relatively unchanged. Nutritionally, liver glutathione appears to be related specifically to the sulfur-containing amino acids—possibly because it requires just one such essential amino acid for its formation.

Fig. 1 shows the comparative loss of glutathione and xanthine oxidase from the liver during protein depletion. For interpretation,

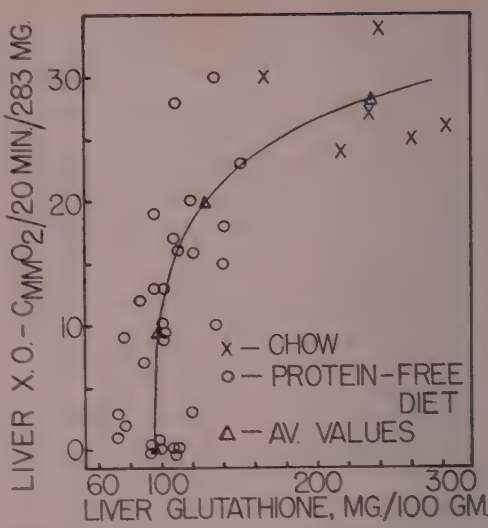

FIG. 1. Comparative loss of glutathione and xanthine oxidase from rat liver during protein depletion. Each symbol represents a liver analyzed simultaneously for reduced glutathione and xanthine oxidase.

the xanthine oxidase axis can be considered a measure of the magnitude of the protein depletion effect on the liver. Most of the labile glutathione was lost before the xanthine oxidase was seriously affected, and all of the labile glutathione was gone when the liver still retained 50% of its xanthine oxidase. Thereafter the residual liver glutathione was maintained constant while the xanthine oxidase was depleted, and when the liver contained less than 10% of its original xanthine oxidase(1), it still retained about 40% of its glutathione.

Xanthine oxidase was found quantitatively in the supernatant fraction when rat liver was homogenized in phosphate buffer and subjected to centrifugation at approximately 30000 x g for 90 minutes(1). A similar analysis for glutathione in the precipitate and supernatant fractions obtained from 4 normal and 4 protein depleted (27 days) rat livers gave the following average results:

	mg glutathione per 100 g original liver	
	Supernatant	Precipitate
Normal rat liver	180	30
Protein depleted liver	68	24

The precipitate of nuclei, mitochondria and

microsomes was not washed in these experiments and the small amounts of adhering supernatant could be expected to increase the glutathione values for the precipitates above their true levels, especially with normal rat livers. Nevertheless, the results were clear in showing that at least 85-90% of the glutathione in rat liver was present in the soluble supernatant fraction, and it was from this fraction that the labile glutathione was lost during protein depletion.

Summary. A protein-free diet reduced liver glutathione in rats to about 40% of its normal value within 1 to 2 days, but did not deplete blood glutathione. The labile 60% of the liver glutathione was lost completely before the liver xanthine oxidase was reduced more than 50%; the residual 40% of the liver glutathione was then retained while the remainder of the xanthine oxidase was lost. 85-90% of the glutathione in rat liver was present in the soluble supernatant fraction obtained by centrifuging an homogenate at 30000 x g.

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Nucleic Acid Metabolism of Bone Marrow and Spleen. I. Normal Values and Effect of Sodium Pentobarbital.* (19272)

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The nucleic acid metabolism of various organs and tumor tissue has been studied extensively(1-4) but only a few isolated observations have been reported on the intact bone marrow(5-7). This organ is of particular interest because of its very active cell division and cell maturation. We have studied the concentration and the relative rate of formation of DNA and RNA in the bone marrow and spleen of the rat. After a tracer dose of radiophosphorus (P^{32}), the DNA and RNA fractions were isolated and determinations made of the phosphorus and P^{32}. The amount of phosphorus in each fraction is a measure of the nucleic acid content, and the % of injected P^{32} per mg phosphorus, or specific activity (SA), an index of the nucleic acid newly formed during the interval studied. Thus the desoxyribose nucleic acid phosphorus (DNAP) is related to the cellularity of the bone marrow and the SA_{DNAP} an index of the rate of cell division. Further, the ribose nucleic acid phosphorus (RNAP) is related to the cytoplasmic content, while the SA_{RNAP} reflects in part the process of cell maturation. This concept is supported by experiments on irradiated rats(8), which show a close correlation between the nucleic acid determinations and the morphologic changes in the bone marrow.

In this paper, normal nucleic acid values are presented and it is shown that anesthetic doses of sodium pentobarbital produce a significant depression of DNA formation in both the bone marrow and spleen.

Methods. White male rats of the Sprague-Dawley strain, approximately 3 to 4 months old, were employed. Their weights varied

* This work was done under contracts with the U. S. Air Force and the Atomic Energy Commission and aided by a grant from Armour Laboratories.

from 265 g to 330 g with an average of 297 g. There was no difference between the average weights of the control and experimental series. The animals received a diet of Rockland Dog Blocks and were fasted for 12 hours before the injection of P^{32}. The control animals were injected intraperitoneally with 1 microcurie carrier free P^{32}† per 100 g of body weight and sacrificed 4 hours later. This tracer dose apparently does not cause a depression of metabolic activity by irradiation, since the P^{32} uptake of the nucleic acid fractions of the bone marrow and spleen after the injection of 1 microcurie and 2 microcuries per 100 g have agreed within the experimental error of the method. The experimental rats were anesthetized by the intraperitoneal injection of 5 mg of sodium pentobarbital per 100 g weight. Radiophosphorus was given 1½ or 24 hours after anesthetization and the animals were sacrificed 4 hours after the tracer injection. The animals were killed by a sharp blow to the occiput. The femurs and tibias of both hind legs were removed immediately and the bones opened transversely through the cancellous portion with a small circular saw. The remainder of the cancellous bone was curretted out with a scalpel and the marrow expressed intact from the shaft by air pressure from a dry syringe. The marrow content, averaging 120 mg in weight, from the four bones was collected on a clean aluminum foil contained in an ice cold moist chamber. It was weighed on a 500 mg capacity torsion balance with a sensitivity of 1 mg. The entire procedure of removing the marrow and weighing was never longer than 10 minutes. The spleen was removed and a portion from the midsection, weighing approximately 150 mg, was placed on an aluminum foil and weighed with the torsion balance. To obtain total weight, the remainder of the spleen was weighed on an analytical balance with a sensitivity of 0.1 mg. The tissues were homogenized in ice water using a Potter-Elvehjem homogenizer(9). The isolation of DNA, RNA and acid soluble phosphorus (ASP) followed the method of Schmidt and Thannhauser(10) as modified in this laboratory for use with small samples. The phosphorus content of these fractions was estimated by the method of Gomori(11). Using standard counting procedures the P^{32} content was determined on aliquots dried in cup planchets. The methods for fractionation of tissue phosphorus compounds were examined by P and P^{32} analyses during the extraction procedures. The ASP and phospholipid were found to be completely extracted without appreciable decrease of RNA and DNA. The separation of RNA and DNA from the total nucleic acid fraction was found to be satisfactory. Davidson(4) has stated that in the Schmidt and Thannhauser procedure there is contamination of the RNA fraction by phosphoprotein which has a higher specific activity than the RNAP and would give falsely high values for the specific activity of the latter. Precipitation of the inorganic phosphorus in the RNA fraction by the method of Delory(12) did not appreciably change the specific activity. It would appear that the quantity of phosphoprotein or its specific activity does not appreciably affect the SA_{RNAP} in the spleen and bone marrow. The analyses of small spleen sections were compared to values obtained with the entire spleen and were shown to be entirely comparable.

Results. The phosphorus content of the fractions isolated from the bone marrow and spleen is given in Table I. Results are reported in terms of milligrams of phosphorus per gram of wet weight of tissue sample. The P^{32} uptake in each fraction is expressed in terms of specific activity. Specific activity represents the percentage of the injected dose of P^{32} present per milligram of phosphorus in the fraction.

Spleen weights expressed both as total weights and percentage of the body weight of the rat are recorded in Table II. Since the spleens were not weighed in some of the earlier experiments, additional animals were studied for spleen weights only. The periods of 5½ and 28 hours after injection of sodium pentobarbital are the same intervals at which the animals were sacrificed in the nucleic acid experiments.

All the results have been statistically evalu-

† Supplied on allocation from the Oak Ridge National Laboratory, U. S. Atomic Energy Commission.

TABLE I. Phosphorus Fractions in the Bone Marrow and Spleen. Normal values and the effect of sodium pentobarbital.

Group	No. of animals	Phosphorus content, mg/g ASP	RNAP	DNAP	Specific activity ASP	RNAP	DNAP
			Bone marrow				
Control	8	1.003	1.261	1.296	.462	.158	.165
σ		±.181	±.324	±.239	±.064	±.064	±.021
1½ hr*	8	1.011	1.311	1.287	.478	.150	.102
σ		±.192	±.464	±.271	±.104	±.064	±.030
P		<.50	<.50	<.50	<.50	<.50	>.99
24 hr*	7	1.132	1.149	1.255	.434	.170	.157
σ		±.217	±.201	±.088	±.065	±.012	±.020
P		<.50	.70	<.50	<.50	.50	.50
			Spleen				
Control	8	.939	.864	.841	.475	.107	.068
σ		±.082	±.149	±.124	±.093	±.023	±.029
1½ hr*	8	1.177	1.103	1.070	.364	.091	.025
σ		±.357	±.587	±.185	±.059	±.023	±.018
P		.90	.80	.50	.98	.90	>.99
24 hr*	7	1.170	.993	1.147	.415	.120	.043
σ		±.140	±.144	±.217	±.021	±.017	±.028
P		>.99	.90	.40	.90	.70	.80

* Interval between administration of sodium pentobarbital and P^{32}. All animals sacrificed 4 hr after P^{32} injection.

ated. The significance of changes in the experimental animals as compared to control animals were subjected to "t" analysis by the method of Fisher(13). Changes were considered significant if the probability was 0.95 or greater. Standard deviations (σ) and probability values (P) are given in each table.

Discussion. In these experiments the specific activity values are considered to be a measure of the relative renewal rate of nucleic acid fractions. The absolute renewal rate cannot be determined because the immediate precursor of the nucleic acid phosphorus and its specific activity is unknown. The precursor is probably in the ASP fraction(4,14).

In a preliminary report from this laboratory (6) values for the DNAP and RNAP content of the intact bone marrow and the rate of incorporation of radiophosphorus into these fractions were presented. The high values of the nucleic acid content and specific activities of the fractions in the bone marrow reported here parallel the findings of other authors (5,7). Our results in general confirm previous studies on the spleen(1-4) and indicate a lower DNAP and RNAP content, and a slower renewal rate for these fractions in the spleen than in the bone marrow.

TABLE II. Spleen Weights. Normal values and the effect of sodium pentobarbital.

Group	No. of animals	Avg wt (g)	Avg % of body wt
Control	14	.794	.25
σ			±.06
5½ hr*	12	1.013	.34
σ			±.05
P			>.99
28 hr*	12	.650	.24
σ			±.05
P			<.50

* Time after administration of sodium pentobarbital.

The SA_{DNAP} and SA_{RNAP} of the bone marrow are essentially equal while in the spleen the SA_{RNAP} exceeds the SA_{DNAP}. This probably reflects a similar degree of cell division and maturation in the bone marrow and a relatively lesser degree of cell division in the spleen.

During the 5½-hour-period following the administration of an anesthetic dose of sodium pentobarbital, there is a statistically valid decrease in the SA_{DNAP} of both the bone marrow and the spleen. The SA_{DNAP} returns to normal in 24 hours. There is no significant change in the nucleic acid concentration or in the SA_{RNAP}.

The increase in spleen weight observed has been reported in other animals(15,16). Since

the DNAP and RNAP per gram tissue do not change significantly, it would appear that the total nucleic acid content of the spleen increases after anesthesia. Recalculation of the per cent of injected P^{32} present in the total nucleic acid in the spleen still shows a depression of the SA_{DNAP} and no significant change in SA_{RNAP}. Thus, it is suggested that an anesthetic dose of sodium pentobarbital temporarily inhibits the rate of cell division in the bone marrow and the spleen. The increase in size of the spleen along with an elevation of the total nucleic acid content are compatible with the sequestration of nucleated cells from the peripheral blood.

Other workers(17,18) have reported reduction in nucleic acid synthesis by the barbiturates. The mechanism by which barbiturates affect cellular metabolism is obscure. Quastel and his associates(19) were the first to demonstrate that barbiturates were capable of inhibiting enzyme systems. Grieg(20) subsequently offered indirect evidence that barbiturates interfere with the mechanism of the hydrogen transport system. More recently Persky, *et al.*(21) and Brody and Bain(22) have suggested that the barbiturates interfere with the normal formation of ATP. This latter compound may be the precursor of DNAP.

Summary. (1) Normal values are presented for the desoxyribose nucleic acid phosphorus and ribose nucleic acid phosphorus as well as the rate of uptake of P^{32} by the DNA and RNA in the bone marrow and spleen of adult rats. (2) Anesthetic doses of sodium pentobarbital were found to produce a significant decrease in the relative rate of formation of DNA in the bone marrow and spleen during a period of 5½ hours. (3) The significance of these observations is discussed.

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Influence of Cortisone upon Acute Inflammation.* (19273)

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Several workers have reported that the formation of granulation tissue is inhibited in animals treated with cortisone(1-5). This resulted in delayed healing of wounds. These findings suggested the desirability of determining whether cortisone retards the processes of acute inflammation. Accordingly a series of experiments was planned to secure evidence bearing upon this question.

Thermal injuries were chosen as the simplest form of uninfected lesions and best adapted to the purpose of these experiments. Such lesions were made upon the abdominal skin of guinea pigs weighing from 350 g to 400 g. In one series, burns were made by applying the mouth of a test tube, 8 mm in diameter filled with water at 90° to 95°C, to the skin for 10 seconds. In another series a pencil of solidified CO_2 (dry ice) about 6 mm in diameter was applied to the skin for 30 seconds. Such lesions were made in two groups of guinea pigs: one group treated with cortone®,† another group serving as controls. In each group, lesions of 24 hours, 6 hours, and 1 hour duration were made, after which the animals were killed under ether anesthesia and sections of the lesions were prepared for histologic study. Each of the treated guinea pigs received 2 injections of 5 mg cortone acetate intramuscularly on the day preceding the experiments and 2 such injections on the day of experiments. Twelve lesions each of 24 hours, 6 hours and 1 hour duration, respectively, were made by heat in cortone-treated animals, and the same number in untreated controls. Eight lesions of the same durations were made by freezing on cortone-treated animals and on 8 untreated controls. A total of 60 lesions in cortone-treated animals was compared with the same number of lesions made in untreated animals under identical experimental conditions.

Results. A prompt inflammatory reaction developed in all instances after the application of heat or cold. The latter provoked a milder reaction. Lesions of 1 hour duration consisted of hyperemia, slight edema and accumulation of leukocytes in the pre-capillaries, capillaries and venules. A few leukocytes were observed in the tissue spaces; diapedesis and occasional capillary hemorrhages were seen. These features progressed in degree and reached almost maximum intensity in 6 hours in the lesions made by freezing. There was further progression in the injuries made by heat.

Acute inflammation includes several major features, *i.e.*, hyperemia, diapedesis of red cells, capillary hemorrhages, edema and leukocytic infiltration, which vary in degree. It seemed appropriate to estimate the degree of each feature separately in each lesion examined. A marked degree of either feature was recorded as 3, a moderate degree as 2, a slight degree as 1, and absence of that feature was recorded as zero. While such a record of results is not mathematically precise, it provides a practical means for the required comparisons. Diapedesis and capillary hemorrhages were combined as one feature. Table I is a tabulation of results recorded by this method.

The sum of the figures in each column indicates the total degree of each feature in that group. By comparison, it is apparent that the degree of each item was less in the cortone-treated animals than in the untreated controls. This difference was greater in diapedesis plus hemorrhages and in edema than in the two other items. The results indicate also that the severity of the inflammatory reaction was greater after burns than after freezing, probably because the latter caused less severe damage to the tissues.

* Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinions or the policy of the Veterans Administration.

† Merck & Co. supplied cortone® used in these experiments.

TABLE I. Tabulation of Results.

Animal	24 hr				6 hr				1 hr			
	Hyperemia	Diapedesis	Edema	Infiltration	Hyperemia	Diapedesis	Edema	Infiltration	Hyperemia	Diapedesis	Edema	Infiltration
Burns: Cortone-treated												
23	2	0	1	1	2	1	1	2	1	0	1	1
24	3	3	2	1	3	3	1	3	2	1	0	0
28	3	3	1	1	3	2	1	1	2	0	0	1
29	3	3	1	0	2	2	1	2	2	1	1	0
35	2	1	2	1	3	1	0	1	3	0	1	0
38	3	3	2	2	2	1	2	2	2	0	1	1
39	2	2	1	2	2	1	2	2	1	0	1	1
42	3	3	1	1	3	1	1	2	2	1	1	1
51	2	0	0	1	2	2	2	1	2	0	1	1
52	2	1	1	1	2	2	2	2	2	1	1	1
53	1	0	3	3	2	1	2	2	1	1	1	0
57	2	0	3	1	1	0	3	1	2	1	3	1
Total	28	19	18	15	27	18	18	21	22	6	12	8
Burns: Untreated controls												
14	3	2	2	1	2	2	3	2	2	1	2	0
15	2	2	2	2	2	1	3	3	2	1	1	0
16	2	1	2	2	3	3	2	2	2	2	2	2
25	3	3	3	2	3	3	2	2	2	1	2	0
36	3	3	3	2	3	3	3	2	2	2	2	0
37	3	3	3	2	3	3	2	2	2	1	2	1
40	3	3	3	2	3	3	3	2	2	1	2	0
41	3	3	2	2	2	2	2	3	2	1	2	1
54	2	2	3	1	3	2	3	3	3	2	2	2
55	2	1	3	2	2	2	3	3	3	2	3	1
56	2	2	3	2	3	3	3	2	2	1	3	1
58	3	3	3	2	3	2	3	2	2	2	3	2
Total	32	28	32	22	32	29	32	28	26	17	26	10
Freezing: Cortone-treated												
20	1	0	1	1	2	0	1	1	0	0	0	0
21	1	0	1	2	0	0	0	0	0	0	0	0
26	2	0	2	2	0	0	0	0	0	0	0	0
27	2	0	2	2	0	0	1	2	0	0	0	0
30	1	0	1	1	0	0	2	2	0	0	1	0
31	0	0	1	1	1	0	2	2	0	0	1	1
35	2	2	1	3	2	1	2	2	2	0	2	1
42	1	0	2	2	1	0	3	3	1	0	1	1
Total	10	2	12	14	6	1	11	12	3	0	5	3
Freezing: Untreated controls												
19	1	0	2	3	1	0	3	3	1	0	1	0
22	1	1	2	3	2	0	3	3	1	0	3	3
32	1	1	3	3	2	0	3	3	1	0	2	2
33	1	0	3	3	2	0	3	3	2	0	2	1
36	2	1	2	3	3	1	3	3	2	0	2	1
37	3	3	3	3	2	0	3	3	2	0	2	1
72	2	1	1	3	2	1	2	2	1	0	1	1
73	1	0	1	1	1	0	2	1	1	1	2	1
Total	12	7	17	19	15	2	19	21	11	1	15	10

Physiologists have shown(6) that adrenal cortical steroids reduce capillary permeability or, in other words, they increase capillary resistance; hence it follows that under the influence of cortisone the passage of fluids from the blood into the tissues would be reduced, thus reducing the degree of edema. The same principle applies to diapedesis and capillary hemorrhages, assuming that capillary resistance is increased under the influence of cortisone. Benditt *et al.*(7) found that cortisone decreased the permeability of capillaries and lessened the spreading reaction which results from hyaluronidase. On the other hand, Menkin(8) reported that cortisone acetate injected intradermally caused an *increase* in capillary permeability. However, he intimated that this might be due to some substance present in the vehicle rather than to the cortisone itself. Michael and Whorton(9) recorded that the leukocytic infiltration, the formation of fibrin and the development of edema were strikingly altered in the early phases of acute inflammation in animals treated with cortisone. Robson and Duthie (10) cited the findings of other workers indicating that increased capillary resistance accompanies the "alarm reaction" in which cortical hormones are important factors. These observations were made following stress of various origins including heat, cold, ultraviolet light, x-radiation, nitrogen mustard, histamine, protein shock, insulin shock and others. These authors tested capillary resistance by applying a vacuum cup to an area of skin and determining the degree of negative pressure necessary to cause capillary hemorrhages in the skin. In cases of rheumatoid arthritis, spondylitis, lupus erythematosis and thrombocytopenic purpura under treatment with ACTH, they found that a definite increase in capillary resistance had occurred.

In view of these observations it is not strange that diapedesis, hemorrhages and edema after thermal injuries, were less in degree in animals which had received cortisone than in the controls. Increased capillary resistance may be a factor also in the degree of hyperemia resulting from tissue damage. The minute vessels may maintain their tonus and not dilate so readily or so extensively as under normal conditions. Furthermore, the walls of these minute vessels may offer more hindrance to the outward migration of leukocytes than would endothelium whose permeability had been increased. We noted frequently that leukocytes had congregated in large numbers within the lumina of the capil-

laries and venules, but relatively few had infiltrated the adjacent tissues.

Summary. (1) The inflammatory reactions resulting from thermal injuries were compared in cortisone-treated animals with those in untreated controls. (2) Diapedesis, capillary hemorrhages and edema were quantitatively much less in the cortisone-treated animals. Hyperemia and leukocytic infiltration also were less in degree but the difference in these items was not so marked as in diapedesis, hemorrhages and edema. (3) There is acceptable evidence that the corticoid hormones increase the resistance of capillary walls. The authors believe that this effect reduces the degree of each of the features in the inflammatory reaction.

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A Phenomenon of Topical Protection Against Hyperergic Inflammation by a Preceding Local Inflammation. (19274)

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The decrease in reactivity of living organisms in the course of repeated, non-physiological irritations or drug administration is a natural law of wide importance. One form of this is well known by the name of "tachyphylaxis." Following a similar principle, an anaphylactic reaction can be avoided by pretreatment of the sensitized animals with a small dose of antigen, whereafter the animals will tolerate a multiple of the lethal dose of antigen ("anti-anaphylaxis"). This phenomenon is mainly based on the exhaustion of specific antibodies in the tissues of the shock organs(1), and seems to be involved in desensitization. Furthermore, we know that protein therapy, which in itself has inflammatory properties, can also exhibit antiphlogistic effects—this being due to an endogenous discharge of gluco-corticoids through the mechanism of the "alarm-reaction"(2,3) and a consequent reduction of the "inflammatory potential"(4). In addition, the phenomenon of local immunity(5) has been shown by various investigators after a preceding nonspecific local inflammation. Thus, the spreading of topically injected virulent bacteria could

TABLE I. Topical Protection by a Preceding Inflammation Against Hyperergic Reaction. Six animals in each series.

Groups		1st inj., .2 ml, subcut.	2nd inj., 24 hr later, intraper.	Mean inhibition of inflammation in left hind paw, %
I	a	D (1:37.5)	E .5 ml (1:2.25)	97.2 ± 2.8
	b	D (1:37.5)	D 1 ml (1:37.5)	69.7 ± 2.8
II	a	E (1:5)	E .5 ml (1:2.25)	96.4 ± 1.6
	b	E (1:5)	D 1 ml (1:37.5)	100 ± 0
III	a	P (3%)	E .5 ml (1:2.25)	34.8 ± 6.3
	b	P (3%)	D 1 ml (1:37.5)	70.6 ± 9
IV		H (100 TRU)	D 1 ml (1:37.5)	68.8 ± 9

D = Dextran, E = Egg-white, P = Peptone, H = Hyaluronidase.

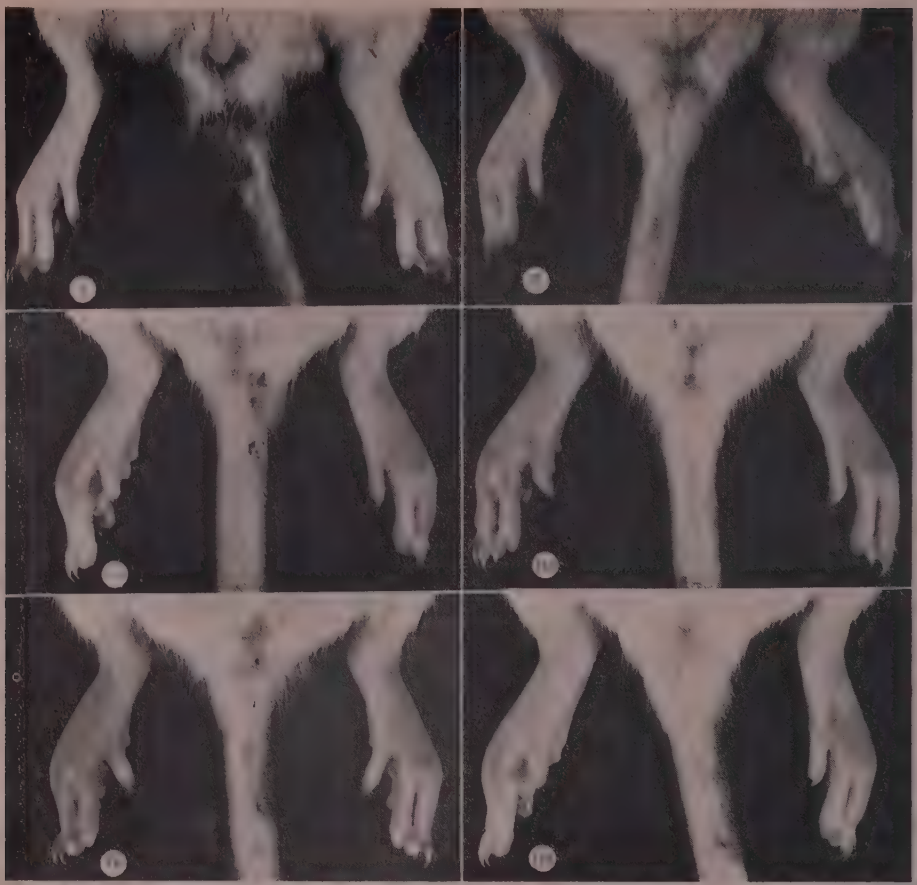

FIG. 1. Protection against dextran- and egg-white-induced hyperergic reaction by a preceding topical dextran or egg-white inflammation in left hind paw of the rat. I—Topical hyperergic reaction: .2 ml dextran (1:37.5) inj. subcut. into left paw (Group I). Ia—General hyperergic reaction 24 hr later: .5 ml egg-white (1:2.25) inj. i.p. (Group I) left paw protected. Ib—General hyperergic reaction 24 hr later: 1 ml dextran (1:37.5) inj. i.p. (Group I) left paw protected. II—Topical hyperergic reaction: .2 ml egg-white (1:5) inj. s.c. into the left paw (Group II). IIa– General hyperergic reaction 24 hr later: .5 ml egg-white (1:2.25) inj. i.p. (Group II) left paw protected. IIb—General hyperergic reaction 24 hr later: 1 ml dextran (1:37.5) inj. i.p. (Group II) left paw protected.

be prevented but also the spreading of color particles, probably by the formation of a fibrinous network (Menkin)(6). As far as we know, however, no studies have been undertaken to investigate the changes in the inflammatory reaction of tissues where nonspecific phlogistic agents are repeatedly administered.

Normally, inflammation proceeds in phases. In the acute phases a subsequent irritation can produce an additional effect. When gross evidence of inflammation subsides, however, we found an obvious resistance against the development of a new inflammation by further administration of an irritant. In the experiments, here described, we have paid special attention to the decrease or elimination of tissue reactivity which is not systemically but strictly locally conditioned.

Methods and results. Following an intravenous or intraperitoneal injection of egg-white(7) or of dextran(8,9) to rats, there develops a strong hyperemia and edema of the snout and paws, which lasts several hours.

A local inflammation of this kind can also be produced by injecting a small amount of egg-white(10) or of other similar agents directly into the paw. We prepared 42 male piebald rats (90-100 g) with a subcutaneous injection of 0.2 ml of various irritants, *i.e.*, dextran, egg-white, peptone and hyaluronidase into the aponeurosis of the left hind paw as shown in Table I. Twenty-four hours later, when the first reaction had disappeared, 6 animals of Groups I-III were given a second injection of dextran intraperitoneally, while the others of Groups I-III received an egg-white solution, also i.p. Another group of animals pre-treated with hyaluronidase received only dextran in the second injection (Table I). These intraperitoneal doses had been proven to produce an inflammatory reaction which was approximately 50% of the possible maximal reaction (9). Two, 3 and 4 fours after the second injection, the intensity of general inflammation was registered in the snout, front paws and right hind paw, and in the pre-treated left hind paw as well.

Depending on the intensity of the first topical reaction, the left paw showed a distinct protection against the action of the second injection, although the latter still produced a general anaphylactoid reaction (Fig. 1). It is pointed out that this protection phenomenon is non-specific. Dextran- and egg-white-induced hyperergic reaction can be inhibited topically by different phlogistic irritants. The preceding inflammation showed a decreasing degree of intensity after egg-white, dextran, hyaluronidase and peptone, in the order mentioned. This is probably why the topical peptone inflammation did not protect to the same extent as the other phlogistic agents, while the topical egg-white inflammation gave the greatest protection.

In order to obtain further data concerning this phenomenon, we studied the duration of the protection: 7 groups of 6 rats each (piebald, male, 90-100 g) were pre-treated with dextran (0.2 ml 1:37.5, injected subcutaneously into the left paw). After 6 hours, the topical inflammation had disappeared almost completely. A second injection of dextran (1.0 ml 1:37.5) was given i.p. as follows:

Groups	8 hr after first injection
I	
II	10
III	14
IV	22
V	38
VI	70
VII	134

The relatively long duration of the protection is shown in Fig. 2. Eight hours after the first injection, residual traces of inflammation in the left paw tended to augment the second inflammation and gave no inhibition. The protection became evident 10 hours after the first injection and increased to a maximum after 38 hours. The decrease of the protection proceeded slowly within the following 90-100 hours.

We are as yet unable to give a definite explanation for the mechanism of this "topical protection phenomenon," and we can only state that it is strictly locally-conditioned.

Summary. (1) The hyperemia and edema of an hyperergic reaction—induced by an intraperitoneal injection of dextran or egg-white in rats—can be topically inhibited by a preceding local inflammation. (2) The degree of this "topical protection phenomenon" depends on the intensity of the preceding inflammation. (3) The preceding inflammation was produced in the paw by a subcutaneous injection of small doses of dextran, egg-white, peptone or hyaluronidase. The phenomenon is therefore considered to be non-specific. (4) Under our experimental conditions the protection phenomenon reached its maximum 38 hours after induction of the first inflammation, and disappeared in the course of ap-

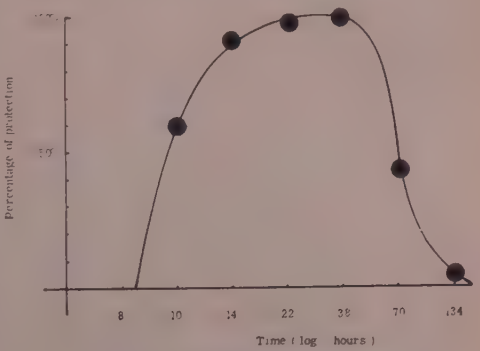

FIG. 2. Duration of protection.

proximately 5 days after the first inflammation.

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Effect of Anti-Rheumatic Drugs on Synovial Membrane Permeability.* (19275)

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Alterations in synovial membrane permeability have been described following the administration of various drugs. Seifter and co-workers(1) found that in rabbits hyaluronidase and desoxycorticosterone (DOCA) produced an increase in the rate of absorption and excretion of phenolsulfonphthalein (PSP) injected into the joint, and that Cortisone, ACTH, and an adrenal cortical extract inhibited absorption and excretion of the dye. They concluded from these studies that one of the controlling factors in membrane permeability is an antagonism between DOCA and Cortisone-like substances. In a later study(2), these investigators reporting on the effects of many steroid substances on synovial membrane permeability suggested that compounds impairing this permeability should produce an anti-rheumatic effect in patients.

Our study was designed to further evaluate the relationship between synovial permeability and the anti-rheumatic effect of cortisone and ACTH in animals and man. We undertook to explore synovial permeability by studying the rate of absorption and excretion of PSP following its injection into a joint. Since Seifter's studies had described its effective use, this appeared to be a convenient and accurate index. However, our results lead us to question the validity of this procedure as an accurate measure of synovial permeability.

Methods and materials. Studies on rabbits were conducted in male animals weighing 3 to 5 kg. Each animal was anesthetized with sodium amytal 100 mg/kg intramuscularly, and hydration was attained by the administration of 25 ml per kg of water by stomach tube. An indwelling catheter was secured in the bladder for collection of urine. PSP was injected into the ankle joint using 0.25 mg per kg, and after the injection, collections of urine were made by irrigation and manual expression of the bladder every 15 minutes for 2 hours. Urine samples were adjusted to a pH of 10.5 and diluted to a standard volume, filtered, and the optical density measured on a Coleman Junior Spectrophotometer at a wave length of 500 millimicrons. Excretion of the dye was calculated for each fifteen minute period as a percentage of the total dye injected. Cortisone was given to 7 rabbits for 11 courses in the doses shown in Fig. 1.

* Aided in part by a grant from the Institute for Study of Sedative and Analgesic Drugs.

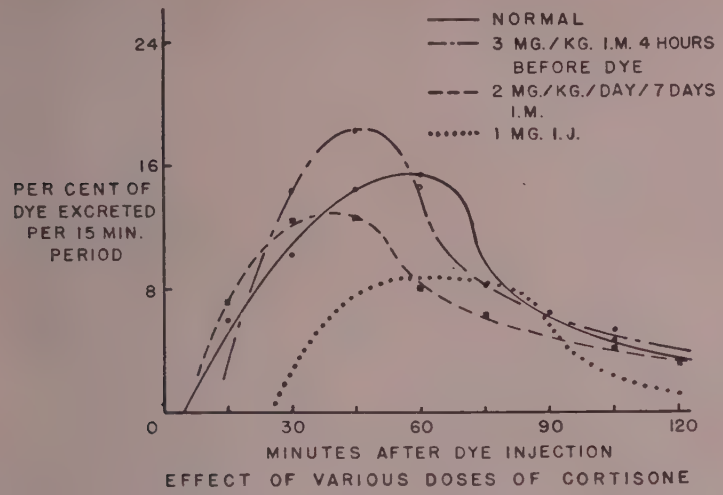

FIG. 1. Absorption and excretion of dye in rabbits. Parenteral cortisone failed to alter normal curve. Cortisone injected into the joint delayed absorption.

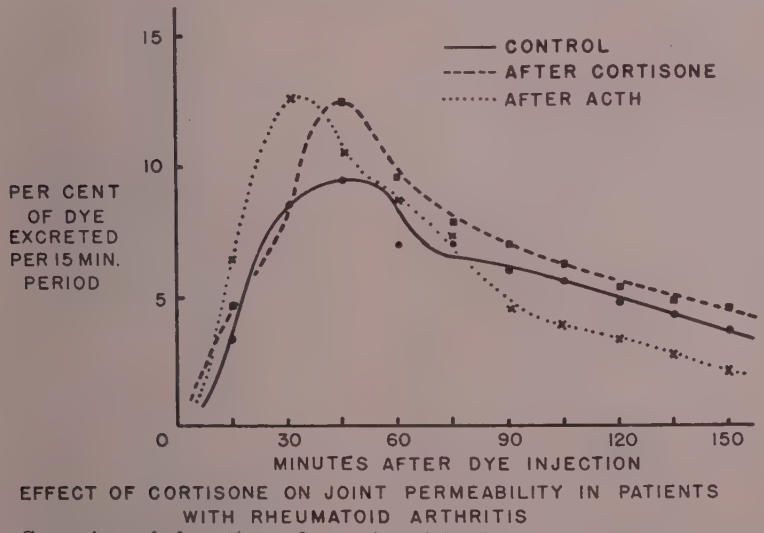

FIG. 2. Comparison of absorption and excretion of dye injected into knee joints of patients with rheumatoid arthritis before and after therapy with cortisone and ACTH.

Three of the animals received 1 mg of Cortisone intra-articularly, and the remainder received it intramuscularly. ACTH was given for 5 courses in 2 rabbits. Renal function was determined by intravenous administration of the dye before and after Cortisone and ACTH therapy. Studies were made of the effects of hyaluronidase administered into the joint along with the dye in 3 normal animals, after Cortisone in 2 animals, and after ACTH in one animal. The presence of dye in the joint was confirmed by careful dissection of the joints of several animals. The method used for investigating synovial membrane permeability in patients was similiar to that outlined above. All patients studied suffered from severe rheumatoid arthritis. In each case the knee joint was injected with 6 mg (1 ml) of PSP and urine collections were made at fifteen minute intervals with a retention

TABLE I. Joint PSP in Rabbits.

Periods		1	2	3	4	5	6	7	8	Total
Control	Avg	5.8	10	15	13.8	8.3	5.8	3.9	3.1	63.3
	SD*	4.6	4.8	4.8	5.6	.7	1	3.8	1	6.7
Cortisone										
3 mg/kg	Avg	0	14	17.5	14	7.5	8	4.5	—	65
3 hr	SD	0	3	2.5	3	2.5	2	.5	—	3
2 mg/kg	Avg	6.5	12	12	7.5	5.5	5.5	4	3	56
7 days	SD	.5	2	2	.5	.5	.5	1	1	6
1 mg IJ	Avg	0	3	5.8	15	7.5	7.5	2	.7	38
	SD	0	2	4.8	4	4	4.7	1	—	8.7
ACTH										
.3 mg/kg	Avg	3	21	13.5	9	4.5	4.5	2	2.5	60
8h-3 days	SD	3	6	4.5	0	2.5	1	2	2.5	7
Hyaluronidase										
150 TRU IJ	Avg	10.8	17.5	14.2	13	9	5	5	3	71.2
	SD	2.3	7	6.2	3	2.5	1	—	—	4
After corti-	Avg	7	13.5	13.5	8.5	4.5	3.5	2	2	54.5
sone	SD	1	.5	.5	1.5	.5	.5	0	0	4.5
After ACTH	Avg	0	13	14.2	9.1	6.4	4	4.1	1	51.8
	SD									

* SD = Stand. dev.; TRU = Turbidity reducing units; IJ = Intra joint.

catheter in the bladder. Adequate oral fluid intake was insured throughout the two and one-half hour period of study. Urine samples were tested, as in the rabbit studies, to determine the percentage of dye in each 15 minute sample. Initial clinical observations included degree of joint deformity, blood counts, sedimentation rates, and synovial permeability to PSP. Cortisone was given to 9 patients for a total of 12 courses in a dose schedule of 300 mg intramuscularly the first day, then 100 mg daily for a total of 14 days at which time studies were repeated. Identical studies were performed on 3 patients before and after a course of ACTH consisting of 20 mg every 6 hours for 14 days. Renal function was determined before and after administration of ACTH and Cortisone in all patients, and no change was noted.

Results. Excretion of the dye following its intra-articular administration to normal untreated rabbits reached a maximum output between 45 and 60 minutes, and excretion was not altered by treatment of animals with Cortisone in doses of 2 mg/kg for 7 days or 3 mg/kg for 3 hours before the study. (Fig. 1) However, 1 mg of Cortisone injected into the joint with the dye resulted in a retardation of its excretion. ACTH in doses of 0.3 mg/kg every 8 hours for 3 days also failed to alter excretion of the dye. Renal function was not inhibited by Cortisone or ACTH.

The administration of 150 turbidity reducing units (TRU) of hyaluronidase into the joints of normal animals resulted in a probably insignificant increase in the rate of excretion of the dye. In animals treated with ACTH or Cortisone, studies of the effects of hyaluronidase showed little alteration in the rate of excretion. Total amounts of dye excreted over the 2-hour period bore no correlation to therapy.

In patients with rheumatoid arthritis, the clinical response to Cortisone and ACTH therapy was marked, but renal excretion of PSP was not altered by therapy. Excretion of PSP injected into the joint reached a maximum in 45 minutes, and was unaltered by Cortisone and ACTH therapy. White blood counts, sedimentation rates and eosinophil counts showed the expected response(3) to therapy with these two substances, but the magnitude of clinical response to anti-rheumatic therapy did not correlate with even minimal variations in the synovial permeability as measured by PSP.

Discussion. The report of Seifter described definite changes in the synovial membrane permeability of rabbits. Hyaluronidase accelerated and ACTH or Cortisone retarded dye

TABLE II. Joint PSP in Patients.

Periods		1	2	3	4	5	6	7	8	9	10	Total
Cortisone												
Pre-treatment	Avg	3	8.3	5.8	6.7	6.7	6	5.3	5	4.2	3.6	56.7
	SD*	2.4	5.5	5.6	3.2	3.6	2.8	3.3	2.6	1.5	2	26.2
Post	Avg	5	7.7	12.3	9.3	7	7	6	5.2	4.2	4	69.1
	SD	5.4	4.5	10	5.7	2.4	2.9	1.9	1.3	2.1	2.6	20.2
ACTH												
Pre-treatment	Avg	3	11.3	10	14	7	6.3	5.3	5.2	3.8	3.8	68.3
	SD	1.6	3.7	3.2	7.5	2.8	.9	.9	.9	.5	.9	9.4
Post	Avg	7	12	10	8.5	6.7	4.8	3.9	3.6	2.7	2.5	65
	SD	3.3	2.8	.9	.8	.9	1.2	.3	.4	.4	.6	8.3

* SD = Stand. dev.

absorption and excretion. From these results he inferred that permeability studies provided an accurate measure of the anti-rheumatic action of certain drugs. Considering the selective permeability of synovial membranes to naturally occurring joint substances (proteins, electrolytes, etc.)(4), it would seem that permeability to PSP might be an inappropriate measure of synovial membrane change. In view of the known histologic changes of rheumatoid arthritic joints following ACTH and Cortisone therapy(5) and the lack of changes in permeability to PSP, our results further suggest that this agent is an inadequate measure of synovial membrane permeability. For greater accuracy, we chose to collect urine at fifteen minute intervals and to calculate the percent of the administered dye excreted during each period rather than record only the times of appearance, peak excretion, and disappearance of the dye. The possibility of depression of renal excretion of PSP by ACTH or cortisone has been eliminated by kidney function studies. The membrane permeability to PSP was not altered to any significant degree in those animals treated with ACTH or Cortisone. The one exception to this statement occurred in 3 animals given Cortisone directly into the joint. We believe that the injection of particulate matter such as Cortisone could have mechanically altered permeability, and we are unwilling to draw conclusions from this limited study. Hyaluronidase injected intra-articularly in the rabbits resulted in a questionable increase in PSP dye excretion. Previous administration of ACTH or Cortisone did not alter this effect appreciably.

The human experiments closely paralleled those done in rabbits. The failure of ACTH and Cortisone to alter joint permeability in these patients was equally apparent regardless of marked clinical improvement.

Summary and conclusions. (1) Permeability studies were performed in rabbits and in patients with rheumatoid arthritis using the method of Seifter which employs injection of PSP into the joint, and recovery of the dye from the urine. (2) Cortisone and ACTH were given to rabbits and patients to determine any effect upon synovial membrane permeability and to detect any anti-hyaluronidase effect in rabbits. (3) Our results fail to demonstrate significant alterations of synovial permeability in rabbits or humans following parenteral therapy with ACTH or Cortisone. There was no evidence of hyaluronidase inhibition in rabbits. (4) Cortisone injected into the joints of rabbits impaired the absorption of PSP. A mechanical blocking effect was not excluded. (5) The injection of PSP into joints is not necessarily a valid measurement of permeability of synovial membranes to physiologic substances.

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Enhancement of Bacterial Infection by Homologous Gastric Mucin.* (19276)

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Although most of the work on enhancement of infection by mucin has been done with hog gastric mucin, mucins from other sources have been used. These include human salivary mucin(1,2), human gastric mucin(3), hog intestinal mucin(4), and guinea pig intestinal mucin(5). In more recent work, mucins from predominantly human sources have also been prepared and assessed for their enhancing activity. These include respiratory tract mucin(6), and mucins prepared from nasal mucus, bronchiectatic fluid, pseudomucinous cyst fluid, stomach, small and large intestine, and umbilical cord(7). However, no work has been reported on the effect of mouse gastric mucin or from mucins of sources other than those mentioned above. In addition, although the guinea pig intestinal extract used by Cantacuzene and Marie(5) was used in guinea pigs with the cholera vibrio as test organism, no other work has been done with a mucin homologous to the animal in which enhancement was being tested. Since mice have been used in work reported elsewhere(8) the preparation of mouse gastric mucin and its enhancing activity was investigated.

Methods. Preparation of the mouse gastric mucin. The method for the preparation of mouse gastric mucin was that described by Bendich, Kabat, and Bezer(9) for the preparation of mucin from individual hog stomach mucosae. From this material Bendich *et al.* (9) extracted "pure" group A substance by the method of Morgan and King(10). The method consisted of peptic digestion of the hog stomach mucosae at pH 2.3, and precipitation of the mucin from the aqueous peptic digest by 4 volumes of 95% ethanol. The stomachs of mice were removed, opened and their contents expressed. They were then placed in 70% ethanol for 10-14 days until enough were available for the next step. Five hundred stomachs, per batch, were washed with water, chopped into small pieces, washed twice with 95% ethanol and once with ether, and dried over P_2O_5 *in vacuo*. The dried stomachs were weighed, and 8.0 ml per g of HCl-citrate buffer at pH 2.3 added. To the suspension was added 2.0 mg crystalline pepsin and 0.2 ml toluene and the mixture was incubated at 37°C for 4-5 days with occasional shaking. The pH was then carefully readjusted to 2.3 with concentrated HCl and 1.0 mg pepsin was added to the mixture. After incubation for 2 more days, the peptic digest was separated by centrifugation. The sediment was suspended in HCl-citrate buffer (4.0 ml per g dried stomachs), 1.5 mg pepsin and 0.2 ml toluene added, and the mixture was incubated for 2 days. It was then centrifuged and the supernate was added to the previous digest. The sediment was washed twice in 60 ml citrate buffer. The combined supernates and washings were clarified by filtration through absorbent cotton. Four volumes of 95% ethanol were added to the clarified, viscous digests, and a heavy, very viscous, white precipitate settled out. This was collected by filtration upon a Buchner filter and after being washed twice with 95% ethanol and once with ether, the precipitate was dried over P_2O_5 *in vacuo*. The resulting grayish-white material was ground in a mortar and pestle, and weighed. This material was the mouse gastric mucin. It was not soluble in either saline or water but would make a stable suspension in either fluid when shaken for 24 hours on a Kahn shaker. Two thousand mouse stomachs were extracted in 4 batches to give a total yield of 2.5 g of the mouse mucin, which represented 4.1% of the weight of the dried stomachs.

"Virulence enhancing" activity of mouse gastric mucin. A stable suspension of the mouse mucin was made by adding 780 mg to 36.0 ml distilled water and shaking on a

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TABLE I. Enhancing Activity of Mouse Gastric Mucin on *Salmonella typhi* Infection in Mice.

No. of organisms	2% mouse mucin*	2% hog mucin	Saline	No. of organisms	5% mouse mucin†	5% hog mucin	Saline
9.3×10^7	—‡	—	4/5	1×10^8	—	—	4/5
10^6	4/5§	—	0/5	10^7	—	—	0/5
10^5	3/5	—	—	10^6	5/5		
10^4	0/5	—	—	10^5	5/5		
10^3	0/5	4/5	—	10^4	4/5		
10^2	0/5	1/5	—	10^3	4/5	4/5	
93	0/5	0/5	—	10^2	4/5	3/5	
0	0/5	0/5		10	—	1/5	
				0	0/5	0/5	

* Heat-sterilized. † Not heat-sterilized. ‡ Not done. § Deaths/total, 36 hr after inoculation. ‖ 28 hr after inoculation.
All mice were observed 5 days before being listed as survivors.

Kahn shaker for 24 hours until homogenization occurred. The resulting suspension (approximately 2% w/v) was a grayish, translucent, viscous material which bore little resemblance to a white, opaque, viscous 2% hog gastric mucin suspension. Both mucin suspensions were sterilized at 121°C for 15 minutes. The mouse mucin precipitated with the heat treatment, but was resuspended easily when it was cooled and neutralized with N/10 NaOH. The hog mucin remained stable through the heating and the neutralization thereafter.

The materials were titrated for activity as shown in Table I. Mouse mucin enhanced infection but was not as effective as the 2% hog mucin suspension.

Since the mouse mucin seemed more heat-labile than the hog mucin and since it was possible that the heat had destroyed some of the biological activity, a 5% suspension of the mouse mucin was prepared aseptically in water at pH 7.3. The preparation was sterile. A titration of its activity in comparison to 5% hog mucin is also shown in Table I. This preparation of mouse mucin was as effective as the hog mucin in enhancing infection by *S. typhi*.

Summary. Homologous mucin will induce the phenomenon known as enhancement of infection in mice as well as the more routinely used hog gastric mucin.

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Relationship of Protein Intake to Protein Anabolic Activity of Testosterone Propionate.* (19277)

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Androgens stimulate an increase in body weight and a positive nitrogen balance in various species (1,2). Preliminary studies in the rat (1a,2) indicated that these phenomena could not be enhanced by an increase in the protein intake or by supplementation with cystine. Further data are presented.

TABLE I. Composition of Diets.*

	I	II	III
Casein	15.7	27.9	46.4
Sugar	61.2	50	31.5
Hydrogenated vegetable oil	7.4	7.4	7.4
Wesson's salts	3.7	3.7	3.7
Cellulose	1.8	1.8	1.8
Yeast	9.2	9.2	9.2
N, %	2.95	4.39	6.80
Protein (N X 6.25), %	18.4	27.5	42.5

* Each rat received 3 times per week 2 drops of cod liver oil and one drop of 34% tocopherol concentrate from wheat germ oil† diluted 10 fold with Wesson oil.

† Provided by Distillation Products Incorporated through the courtesy of Dr. Philip L. Harris.

Method. The rats were purchased from the Carworth Farms and weighed 240 to 300 g. They were castrated at 300 to 350 g body weight, brought to body weight and nitrogen equilibrium while eating the 18% protein diet and transferred to their respective diets (Table I) at 60 days prior to the first experiment. The testosterone propionate,‡ a 10 mg per ml solution in sesame oil, was injected subcutaneously at the same time each day for 21 days. The experimental and analytical procedures have been described (3).

Results. The characteristics of the changes in body weight and nitrogen balance during and subsequent to the injection of the androgen were not altered by the changes in protein intake (3).

The increases in protein intake had no

TABLE II. Protein Anabolic Activity of Testosterone Propionate in Castrated Rat at Different Levels of Protein Intake.

Protein in diet, %	Rat No.	Body wt.		Urine,	Nitrogen	
		Pre-injection, g	Max. inc., g	pre-inj., mg/day	Max. retained/day,* mg/day	Total retained, mg/21 days
		T. P. at .25 mg/day				
18	5	343	13 ± 1.5†	248	48 ± 6†	758 ± 121†
28	4	356	12 ± 1.8	353	50 ± 12	958 ± 160
43	5	353	11 ± 1.7	570	62 ± 8	1048 ± 108
		T. P. at .50 mg/day				
28	5	348	12 ± 1.7	352	51 ± 8	740 ± 137
43	5	321	15 ± 1.6	593	57 ± 5	820 ± 115

* Average of 2nd, 3rd, and 4th periods (7 days) when N retention was maximum.

† Standard error of the mean $= \sqrt{\frac{\Sigma X^2 - \bar{X}^2}{N}} \Big/ \sqrt{N-1}$

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effect on the body weight stimulating effect of the androgen (Table II). The maximum daily nitrogen retention and total nitrogen retained also were not significantly changed ($P < 0.05$). Furthermore, an increase in the dose of the androgen (Table II) also did not produce any greater anabolic effects at either the 30 or 50% levels of protein intake.

Discussion. Once an adequate protein intake is provided a further increase in the protein intake of the rat by the replacement of part of the carbohydrate of the diet by an isocaloric amount of protein (casein) does not enhance the over all protein anabolic activity of testosterone propionate. Stimulation by androgen of the retention of nitrogen or growth processes apparently is regulated by inherent properties of the various cells.

Summary. The protein anabolic activity of testosterone propionate in adult male castrated rats was not altered by increasing the protein content of the diet from 18 to 28 and 43% by replacement of carbohydrate.

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New Inhibitors of Enzymatic Proteolysis.*† (19278)

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Earlier reports from this laboratory(1,2) described the inhibition of pepsin, trypsin, papain, and cathepsins by several carbonyl group reagents. Since then, additional inhibitors of proteolytic processes have been found. The new inhibitors (guanidine, arginine, ethylenediamine, lysine, and others) show certain structural similarities to the compounds tested before, but they are not carbonyl group reagents.

Most of the new inhibitors were effective only when egg-white served as substrate and would not have been discovered if the current preference for synthetic substrates had been strictly adhered to. The observation that certain inhibitors of the proteolysis of natural substrates fail to interfere with the enzymatic hydrolysis of synthetic substrates, demonstrates that results obtained with well-defined synthetic systems are not necessarily applicable to more complex and more natural systems.

A. Experiments with egg-white as substrate. The results of these experiments are listed in Table I. Tests with a few aldehyde reagents and with pyrophosphate were carried out simultaneously for purposes of comparison. The substrate was prepared as described before(1) and the progress of hydrolysis was followed photoelectrically(1).

B. Experiments with casein as substrate. (a) Substrate for tests with pepsin—same as described before(1). *(b) Substrate for tests with trypsin and chymotrypsin*—a mixture of 2 g casein and 200 ml phosphate buffer ($M/15$; pH 8.30) was heated in boiling water for 15 minutes. After cooling, 20% NaOH was added dropwise until pH 8.4 was reached. *(c) Incubation mixtures*—to 4 ml substrate was added 1 ml water or 1 ml inhibitor solution, which had been adjusted to the pH of the incubation mixture with HCl or NaOH. The mixture was kept at 37°C for 10 minutes.

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TABLE I. Effect of Inhibitors on Enzymatic Hydrolysis of Egg-White.

Inhibitor	Mole/l	% inhibition after 30 min at 23°		
		Pepsin	Trypsin	Chymotrypsin
[illegible]	.10	62	94	84
Creatine	.04	48	25	—
Arginine	.04	58	75	64
Diethyldithiocarbamate	.02	52	50	—
[illegible]	.10	71	88	66
o-Phenylenediamine	.02	—	75	74
	.04	79	—	—
Hydroxylamine	.02	26	55	27
Hydrazine	.02	81	65	38
	.10	—	96	90
Phenylhydrazine	.02	38	61	70
	.10	—	100	95
Ethylenediamine	.02	—	68	—
	.10	77	94	84
Tetracarboxymethyl-ethylenediamine	.02	—	85	65
	.10	—	99	91
Aniline	.02	33	—	—
	.10	—	27	—
Sulfanilic acid	.02	17	—	—
	.04	—	72	37
Sodium pyrophosphate	.01	—	75	62
	.10	60	96	95

Each inhibition test was performed together with a test in absence of inhibitor at the same pH (1.4-1.5 was the range for pepsin, 7.5-7.7 for trypsin, and 8.1-8.2 for chymotrypsin). The results from 6 readings each at 3 min intervals were plotted as pairs of time-hydrolysis curves. The degree of inhibition was then calculated from the inverse time-activity relationship. This method of evaluation was based on the finding that the times required to reach a given degree of hydrolysis were inversely proportional to the amounts of enzyme present. Enzyme concentrations per 100 ml incubation mixture were: cryst. pepsin .33 mg, cryst. trypsin 9 mg, and cryst. chymotrypsin 3.6 mg. The trypsin content of the cryst. trypsin preparation (Armour) was found to be about 4% by weight (protein nitrogen determination and measurement of ultraviolet absorption).

One ml enzyme solution (containing respectively 0.008-0.036 mg cryst. pepsin, or 1 mg cryst. trypsin, or 0.4 mg cryst. chymotrypsin), which had also been warmed to 37°C, was then added. The pH of the incubation mixtures was 1.61-1.63 (pepsin), and 8.1-8.4 (trypsin, chymotrypsin). *(d) Measurement of hydrolysis and calculation of degree of inhibition.* After 30 minutes incubation at 37°C, 10 ml 0.3 *N* trichloroacetic acid were added to each tube. After 10 minutes standing, the mixtures were filtered, and 1 ml portions of each filtrate were pipetted into colorimeter tubes containing 10 ml 0.3 *N* NaOH and 4 ml water. Three ml diluted (1:3) phenol reagent(3) were then added and the intensities of the resulting blue colors were measured using a Lumetron Colorimeter Model 402-E and filter 660. The degree of inhibition (expressed as % decrease in tyrosine containing, trichloroacetic acid soluble fragments) was calculated from the extinction measurements obtained in parallel experiments with and without inhibitors. Preliminary tests, made to ascertain that the inhibitors alone did not react with phenol reagent, removed hydrazine, hydroxylamine, o-phenylenediamine, phenylhydrazine, and Versene (tetrasodium salt of N,N,N′,N′-tetracarboxymethylethylenediamine) from consideration in this procedure. The inhibition of the peptic hydrolysis of casein by hydrazine had been determined previously(1) with the aid of ultraviolet absorption measurements in protein-free filtrates. The same technic was now used to determine the effect of hydroxylamine and of bisulfite on the peptic hydrolysis of this substrate. *(e) Results with pepsin.* Previous data showed(1) that the peptic hydrolysis of casein was inhibited by hydrazine, but that higher concentrations of this inhibitor were needed for this substrate than for egg-white

to give the same degree of inhibition. A similar observation was now made with guanidine as inhibitor. In presence of a guanidine concentration of 0.1 Mole/liter there was observed a 20% inhibition of the peptic hydrolysis of casein. The same concentration of guanidine inhibited the peptic hydrolysis of egg-white about 60% (Table I). Hydroxylamine (0.02 *M*) inhibited the hydrolysis of casein 32%, as compared to 26% after 30 minutes incubation time, when egg-white was used as substrate. Sodium bisulfite (0.002 *M*) inhibited 12%, whereas 8% inhibition were observed with egg-white as substrate. *(f) Results with trypsin and chymotrypsin.* In contrast to the observations with egg-white as substrate, no inhibition was caused by arginine (0.04 *M*), ethylenediamine (0.10 *M*), guanidine (0.10 *M*), lysine (0.10 *M*), and sulfanilic acid (0.10 *M*). Sodium pyrophosphate (0.018 *M*) produced a slight but reproducible inhibition of about 8% of trypsin as well as of chymotrypsin.

C. Experiments with synthetic substrates. (a) Pepsin and carbobenzoxy-L-glutamyl-L-tyrosine. It had been reported previously(1) that hydrazine (0.009 *M*) inhibited the effect of cryst. pepsin (7.3 mg/ml incubation mixture) on this substrate by 23%. Since then, the suggestion has been made that a cathepsin, contained in pepsin preparations, rather than pepsin itself might be inhibited by this agent (4). The experiments were therefore repeated using two different fresh preparations of cryst. pepsin. In 6 experiments, with the same enzyme and inhibitor concentrations as stated above, the inhibition varied from 0-4% (average 2.2%) and cannot be considered significant. Guanidine (0.1 *M*) under otherwise identical conditions, inhibited slightly (9-14%). *(b) Trypsin and benzoyl-L-arginineamide.* The incubation mixtures were prepared by adding 0.5 ml trypsin solution (20 mg cryst. trypsin dissolved freshly for each test in 5 ml water) to a mixture of 1 ml substrate solution (containing 0.05 millimole benzoyl-L-arginineamide in *M*/15 phosphate buffer, pH 7.86) and 0.5 ml inhibitor solution or water. The progress of hydrolysis at 37°C was followed with the Grassmann and Heyde procedure(5). In experiments with lysine and Versene interference by these substances with the observation of a sharp endpoint necessitated the use of an aeration method, similar to that described by Greenstein and Leuthardt(6), for the determination of the splitting of the substrate. In this method, the ammonia liberated by the action of trypsin was trapped in boric acid and measured by titration with HCl.

TABLE II. Inhibition of Tryptic Hydrolysis of Benzoylarginineamide by Guanidine, Lysine, and Arginine.

Inhibitor	Mole/l	pH	% inhibition after 30 min	60 min	2 hr
Guanidine	.05	7.48	23	15	23
	.10	7.45	47	42	41
	.15	7.40	55	55	54
	.20	7.42	60	58	56
Lysine	.10	7.57	19	18	19
Arginine	.04	7.52	7	4	15

First order reaction constants were calculated from the hydrolysis data and used to determine the degree of inhibition. The "constant" found after 2 hours incubation time was markedly lower than the corresponding values after 30 minutes and 1 hour, due to the inhibitory effect of benzoylarginine. The value for the "constant" at a given incubation time was, however, quite reproducible in numerous experiments with and without inhibitors.

The following substances did not inhibit the tryptic hydrolysis of benzoylarginineamide: hydrazine (0.10 *M*), phenylhydrazine (0.017 *M*), hydroxylamine (0.025 *M*), creatine (0.025 *M*), and Versene (0.030 *M*). Inhibition was observed in presence of guanidine, lysine, and arginine as shown in Table II.

D. Search for stable inhibitor-enzyme complexes. (a) Recrystallization experiments. Several samples of cryst. pepsin were recrystallized from either 25% alcohol or from 25% alcohol containing various concentrations of hydrazine (0.02-0.16 *M*). The time-hydrolysis curves obtained with the resulting products and casein as substrate were identical, indicating that the crystalline material was unchanged pepsin. *(b) Dialysis experiments.* Mixtures of enzymes (pepsin or trypsin) and inhibitors (hydrazine, guanidine, ethylenediamine) were dialyzed at pH 2.5-3.0 and samples were tested periodically against egg-white

as substrate. After 23-41 hours dialysis the mixtures, which had contained inhibitors at the beginning of the test, showed full activity, identical with that of enzyme solutions dialyzed at the same pH and 4°C in absence of inhibitors.

Discussion. It was found that, in addition to carbonyl group reagents, other compounds, which are structurally related to these reagents, interfere with enzymatic proteolysis. The diamines hydrazine, ethylenediamine, o-phenylenediamine, and Versene, for example, were found to inhibit egg-white hydrolysis. The effectiveness of Versene illustrates that free amino groups are not essential for inhibitory activity.

Since semicarbazide inhibited pepsin and trypsin(1), other carbamic acid derivatives were tested. Urea (0.10 *M*) was ineffective, but guanidine, and its derivatives creatine and arginine surpassed semicarbazide in inhibitory activity. Thiourea (0.10 *M*) showed no activity, but diethyldithiocarbamate (0.02 *M*) interfered markedly with the peptic and tryptic hydrolysis of egg-white.

As the effectiveness of the inhibitors described in this report depends quite obviously on the nature of the substrate used, one is inclined to assume that not the enzyme but the substrate seems to be influenced by the inhibitors in such a manner that it becomes more resistant to enzymatic attack. It has been shown by a number of investigators that the chemical manipulation of various proteins, such as benzoylation(7), reduction by sodium in liquid ammonia(8), or heating with glucose(9) resulted in resistance toward hydrolysis by one or several proteolytic enzymes. It is possible that some of the inhibitors described in this report act through a similar mechanism, which amounts, essentially, to the change of proteolysis facilitating side chains into less effective derivatives, such as addition products.

Summary. (1) The hydrolysis of egg-white by pepsin, trypsin, and chymotrypsin was inhibited by aniline, arginine, creatine, diethyldithiocarbamate, ethylenediamine, guanidine, lysine, o-phenylenediamine, pyrophosphate, sulfanilic acid, and a variety of carbonyl group reagents. The hydrolysis of this substrate by trypsin and chymotrypsin was also inhibited by N,N,N′,N′-tetracarboxymethylethylenediamine. (2) The peptic hydrolysis of casein was inhibited by guanidine, and by several carbonyl group reagents. The hydrolysis of casein by trypsin and chymotrypsin was not inhibited by arginine, ethylenediamine, guanidine, lysine, and sulfanilic acid. (3) The hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by pepsin was not influenced by hydrazine, but was slightly (9-14%) inhibited by guanidine. The effect of trypsin on benzoyl-L-arginineamide was inhibited by arginine, guanidine, and lysine, but not by creatine, hydrazine, hydroxylamine, phenylhydrazine, and Versene. (4) Dialysis and recrystallization experiments with enzyme-inhibitor mixtures led to the recovery of fully active enzymes. The experimental evidence suggests that not the enzymes but the substrates are influenced by the inhibitors listed above, in such a manner that they become more resistant to enzymatic attack.

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The Mechanism of the Hypotensive Action of Veratrum Alkaloids.* (19279)

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The characteristic action of veratrum alkaloids in producing hypotension, bradycardia and depression of respiration has long been recognized(1,2). The importance of the motor fibers of the vagus in the production of the circulatory component of this so-called "Bezold reflex" has been equally well known (2). It has not been clear, however, whether this reflex action is the entire mechanism whereby veratrum alkaloids produce a persistent lowering of blood pressure in the intact animal. As a matter of fact, there are several reasons to believe that the vagal stimulation may be of secondary importance. For example, it has been shown that after section of the vagi or after the administration of atropine, veratrum alkaloids still produce a dramatic fall in systemic blood pressure(3,4). Furthermore, it has recently been shown that a wide variety of chemical compounds are capable of inducing the Bezold or Bezold-like effect, and yet none of these produce a hypotensive effect after vagotomy(5,6,7).

The availability of several new alkaloids (8,9) and the revival of clinical interest in the possible use of vertrum alkaloids in hypertension has prompted the studies reported here with the hope that they will contribute to a better understanding of the mechanism of action of this group of agents. Since the major part of the work reported in this paper was done with germitrine and neogermitrine,† it also seemed important to determine whether these two new alkaloids behave in a fashion similar to those which had been studied previously. The alkaloids in water or dilute acetic acid solutions (pH 6-7) were injected intravenously over a period of 5-30 seconds.

Action of germitrine in isolated and perfused head preparation. A series of dogs was prepared according to the method of Heymans and Heymans(10) in such a manner that the head of recipient dog B was connected to its trunk only by means of the vagus nerves. Appropriate doses of germitrine and neogermitrine were injected into: a) the carotid arteries of the isolated head, b) intravenously into the intact donor dog A, and c) into the isolated trunk of dog B. Fig. 1 illustrates results from a typical experiment. It is particularly to be noted that germitrine injected into donor dog A produced the reflex circulatory effect only in the intact animal with no response in the recipient dog. Furthermore, injection of germitrine into the isolated trunk induced a typical effect in the recipient dog. Heymans and de Vleeschhouwer(11) have reported with veratridine that bradycardia can be produced not only by stimulation of receptors in the isolated trunk but also by stimulation of the chemo-receptors of the carotid body and by direct stimulation of the vagal center of the isolated and perfused head. It should be pointed out, however, that in the experiments by Heymans and de Vleeschhouwer much larger doses of veratridine were required to induce the typical response from stimulation of the carotid sinus itself or the vagal center than was the case for stimulation of receptors in the trunk. It is possible that higher concentrations of germitrine and neogermitrine might also produce similar results. It seems fair, however, to conclude that with doses of veratrum alkaloids not too different from clinical doses in the intact animal the major part of the reflex circulatory effect originates from stimulation of receptors in the trunk and which travel centrally by way of the vagus.

Fig. 1 is also of interest in that it shows the short duration of the reflex circulatory effect which is produced in the recipient dog; whereas, in the intact animal there is a much slower recovery of blood pressure, suggesting that in the intact animal the persistent hypo-

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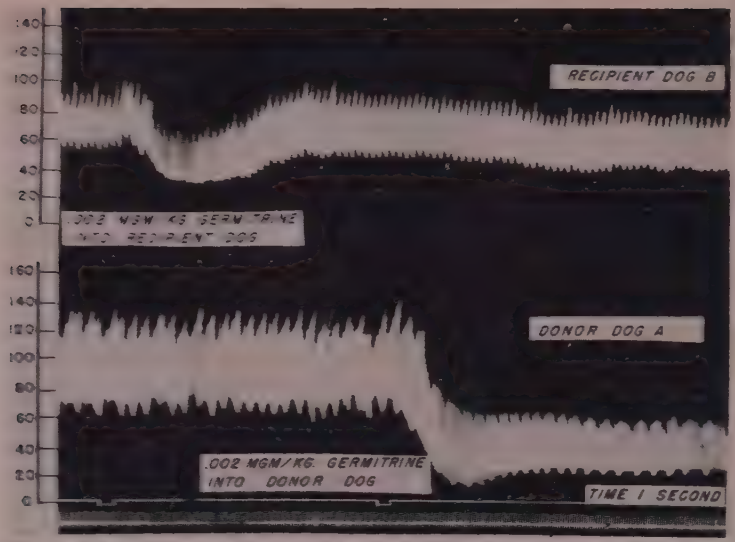

FIG. 1. Effect of germitrine on the vago vaso-inhibitory mechanism of the dog. Animals prepared according to the methods of Heymans and Heymans. Note typical bradycardia induced in recipient dog when drug is injected into trunk but not when same dose is injected into donor dog.

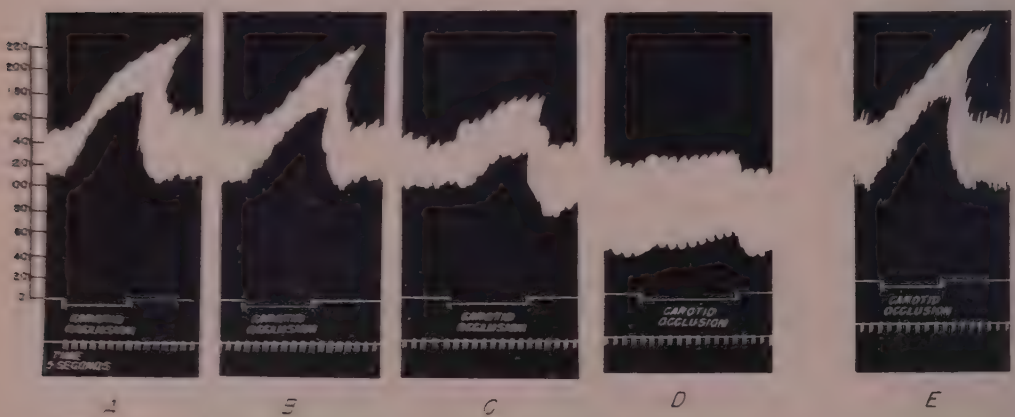

FIG. 2. Typical experiment illustrating effect of germitrine on the carotid sinus pressor reflex of a dog [illegible] with chloralose and morphine, both vagi cut and animal given .5 mg kg of atropine sulfate. A. Carotid response, B. After .0005 mg per kilo, C. After .001 mg per kilo, and D. After .0015 mg per kilo of germitrine, E. Repeat carotid occlusion 1.5 hr after D.

tension is in part due to some phenomenon other that the Bezold effect.

Effects on carotid sinus pressor reflex. In a routine study of the action of veratrum alkaloids, it was found that they possessed a marked ability of blocking the carotid sinus pressor reflex in dogs. Dogs were prepared with chloralose and morphine as described previously (12). After obtaining control observations on the carotid sinus pressor reflex, repeated doses of various veratrum alkaloids were injected intravenously at 5 minute intervals and the response to carotid sinus occlusion tested after each administration of drug. A typical record of such an experiment is shown in Fig. 2 in which germitrine produced a pro-

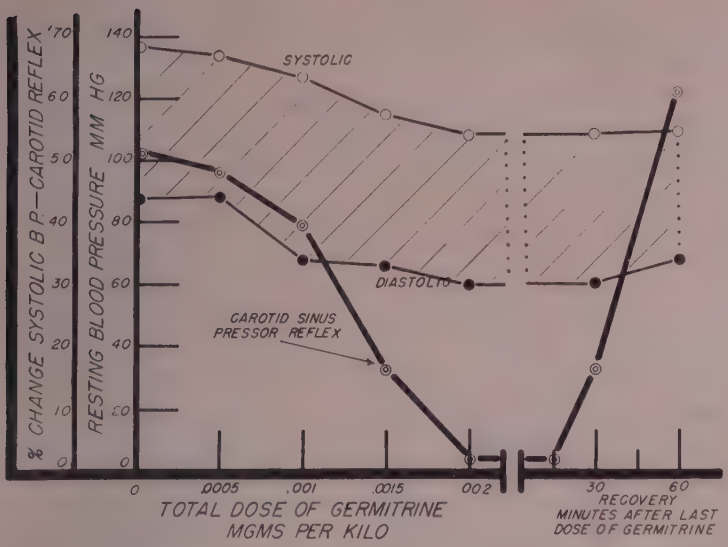

FIG. 3. Summary of effect of germitrine on the resting blood pressure and carotid sinus pressor reflex of dogs anesthetized with chloralose and morphine. Mean results of six separate experiments.

gressive and finally almost complete blockade of the carotid sinus pressor reflex. Following essentially complete blockade animals were tested at intervals until there was recovery of the carotid sinus pressor reflex. Five to 7 dogs were used to determine the activity of each active compound and the results were averaged together as shown in Fig. 3. The dose producing a 50% blockade of the carotid sinus pressor reflex was determined graphically from the dosage-response curve from each series of experiments.

Table I summarizes the results obtained with a series of veratrum alkaloids and semi-purified veratrum preparations. In general, the activity of all compounds tested by this method was similar to that previously reported for other methods of assay(13).

Previously, it had been demonstrated that blockade of the carotid sinus pressor reflex was produced by the following 3 methods: 1) Synaptic blocking agents(12), 2) by a decrease in resting blood pressure(14) or effective blood volume, *i.e.*, hemorrhage(15), and 3) adrenergic blocking agents(16). None of the veratrum preparations used in this study proved to possess either adrenergic blocking or synaptic blocking action. It is possible, however, that the depression of the carotid sinus pressor reflex might have been secondary to a general vasodilatation, either directly or indirectly, since a decrease in resting blood pressure was observed as shown in Table I and Fig. 3. That this seemed unlikely was demonstrated in experiments in which the carotid pressor reflex was blocked by an effective dose of germitrine and despite the infusion of 2-3 liters of 6% dextran solution into several animals, the reflex remained blocked. As additional evidence, it should be pointed out in Fig. 3 that there was practically complete recovery of the carotid sinus pressor reflex in one hour without significant changes in resting blood pressure. Thus, it appears that the blockade of the carotid sinus pressor reflex produced by the veratrum alkaloids can not be explained on the basis of the above-mentioned means of inducing such an effect. Subsequent experiments tend to suggest that an important action of veratrum alkaloids may be on the vasomotor center itself.

Effect on vasomotor center. A series of dogs was prepared according to the technic of Sollmann and Pilcher(17). Animals were anesthetized with chloralose and morphine. Blood pressure was recorded from the femoral artery by means of an Anderson glass manometer. The spleen was mobilized and a major artery cannulated. The corresponding vein

TABLE I. Relative Activity of Veratrum Alkaloids on Carotid Sinus Pressor Reflex.

Compound or preparation	No. dogs used	Dose for 50% blockade of carotid sinus pressor reflex,* mg/kilo	Ratio of activity, germitrine = 1	Time for recovery, min	Mean resting blood pressure, mm Hg systolic/diastolic	
					Before treatment	After carotid sinus blockade
Germitrine	6	1.3 ± .1	1	60	139/90	115/65
Neogermitrine	5	1.6 ± .2	.8	60	153/99	112/69
Protoveratrine	6	3 ± .5	.4	60	130/90	115/80
Germerine	5	4 ± .6	.3	60	152/99	110/65
Germidine	5	4 ± .7	.3	60	152/106	132/89
Veriloid	7	9 ± 1.5	.14	45	135/89	112/75
Veratramine	1	>505	<.0025	—	—	—
Jervine	3	>7000	<.0002	—	—	—
Germine	1	>9333	<.0001	—	—	—

* ± Standard deviation.

was also isolated and cannulated. Immediately following this procedure, infusion of Locke-Ringer's solution into the splenic artery was begun. All other vessels to the spleen were isolated and tied off, care being taken not to include any nerves in the various ligatures. Pressure in the perfusion fluid was obtained by placing the storage bottle of solution approximately three feet above the level of the dog. A Shipley rotameter was inserted into the system at a point just before the perfusing fluid entered the spleen. Flow of perfusing fluid was determined by means of a visual galvanometer which had previously been calibrated. In a properly prepared animal the outflow from the spleen became clear approximately one-half hour after the operation was completed. In order to test the effect of complete isolation of the splenic circulation, epinephrine was injected intravenously. Under proper conditions, this procedure caused no effect on the rate of flow of perfusing fluid in the spleen, although it caused a considerable rise in blood pressure. Both carotid arteries were isolated so that they could be occluded in the usual test for sensitivity of the carotid sinus pressor reflex.

Fig. 4 illustrates the type of results obtained with this preparation. Panel A represents the effects of control carotid occlusion and shows the typical rise in blood pressure and decrease in splenic flow as a result of this maneuver. Panel B illustrates the effects of injecting 0.002 mg per kilo of neogermitrine intravenously into the animal. The typical fall in blood pressure in the systemic circulation is illustrated. Significantly, however, this fall in pressure was accompanied by a vasodilatation of the vessels of the spleen and since the spleen was essentially isolated from the rest of the body except for its nerve supply, it is apparent that this effect must have been mediated by nervous pathways. Panel C represents a repetition of the carotid occlusion 5 minutes after the injection of neogermitrine, illustrating a complete blockade of the carotid sinus pressor reflex as well as no effect upon flow through the spleen. Panel D illustrates the effects of carotid occlusion one hour after the injection of neogermitrine in which instance there had been a partial recovery of the carotid sinus pressor reflex and of the concurrent reflex decrease in splenic flow. Results similar to these were obtained in three dogs each using neogermitrine and protoveratrine.

In order to further eliminate the possibility that the actions described above might have been due to direct effects of the veratrum alkaloids in spite of extreme care in isolating the splenic circulation, neogermitrine and protoveratrine were injected into the perfusate going directly into the spleen. Under such circumstances neogermitrine consistently caused a vaso-constriction rather than a dilatation (Fig. 5). This is of interest in connection with observations of Heymans and de Vleeschhouwer(11) that injection of veratridine into a completely isolated and surviving trunk produces a rise in blood pressure rather than a fall. We have also observed that doses of neogermitrine of the order of 0.01 mg per kilo of this alkaloid tend to produce a rise in blood pressure rather than a fall. Such effects were

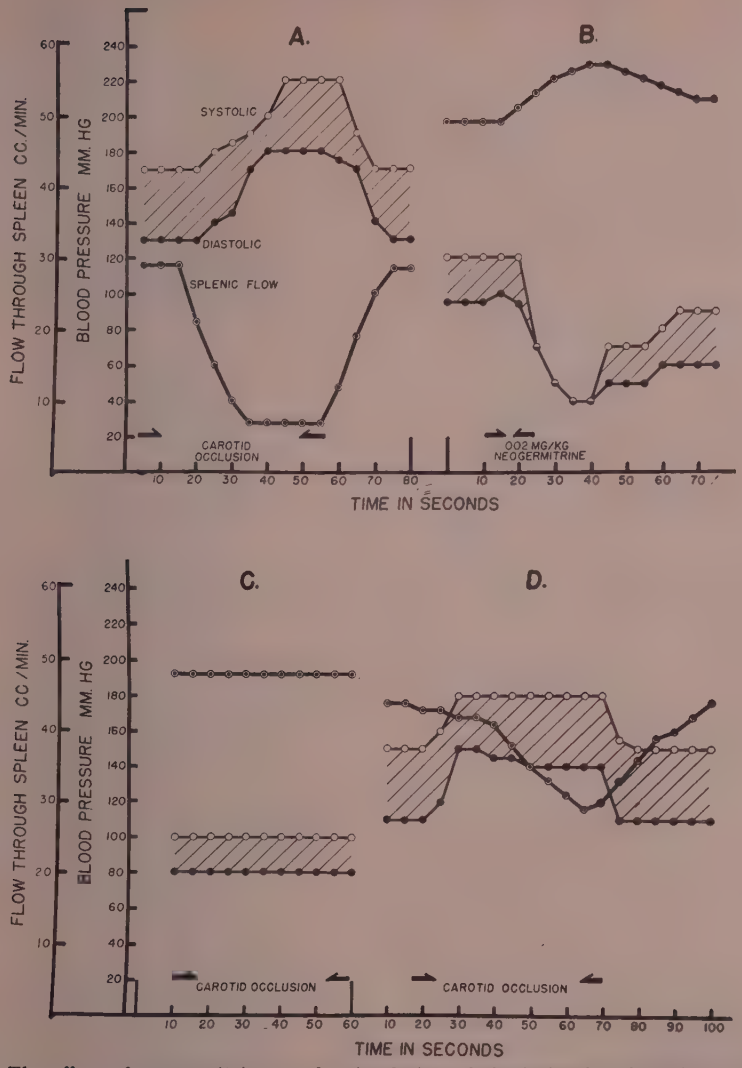

FIG. 4. The effect of neogermitrine on the circulation of the isolated and perfused spleen of the dog prepared according to the technic of Sollmann and Pilcher. Splenic flow determined with a Shipley rotameter placed in perfusion system just before entrance into the splenic artery. A. Effect of carotid sinus occlusion before treatment. B. Effect of intravenous injection of .002 mg per kilo of neogermitrine. C. Carotid occlusion 5 min after B. D. Carotid occlusion 1 hr after B.

not noted with protoveratrine and as shown in Fig. 5, protoveratrine injected directly into the splenic circulation produced little effect upon the rate of flow.

Discussion. The above observations clearly indicate that veratrum alkaloids which have thus far been studied, produce two distinct actions upon the circulation of the dog. One of these is reflex stimulation of the cardio-inhibitory vagal center by means of the Bezold reflex. The second is a blockade of the carotid sinus pressor reflex, which has as its function maintenance of blood pressure at normal levels. The present experiments do not furnish evidence as to the direct site of this blockade, but are suggestive of the point of view that

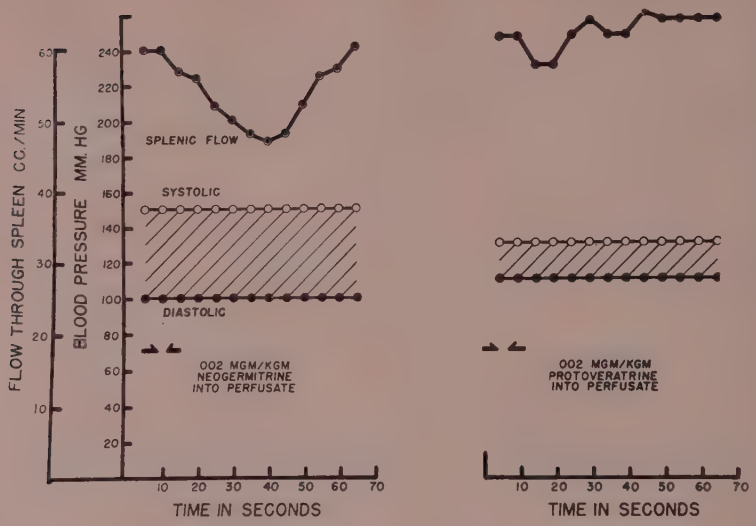

FIG. 5. Same preparation as described in Fig. 4. Injections made directly into the splenic arterial circulation. Note that under these conditions neogermitrine produces a marked vasoconstriction while protoveratrine is almost without effect.

this second action of veratrum alkaloids is directly upon the vasomotor center. Other evidence (18,19) also indicates that the site of the hypotensive action may be central.

The experiments reported here are of interest in another connection in that they suggest the possibility of developing a more precise method of assay of the activity of veratrum alkaloids than is now available. This possibility has been explored by Rubin and Burke (20) and preliminary observations indicate that the method based on the ability of these alkaloids to block the carotid sinus pressor reflex will, in fact, give a more sensitive and more reproducible bioassay result.

Summary. 1. A series of veratrum alkaloids including two new ones, germitrine and neogermitrine, which have recently become available have been studied for their effect upon the circulation of dogs. 2. Germitrine and neogermitrine, as well as other veratrum alkaloids, produce reflex respiratory and circulatory effects. In major part these responses are initiated by stimulation of afferent fibers which run in the vagi-aortic nerves. 3. All clinically active veratrum alkaloids produce a reversible blockade of the carotid sinus pressor reflex. Activity upon this particular reflex corresponds approximately to the reported therapeutic action of these agents in hypertension. It is suggested that this may be made the basis of bioassay procedures for this group of substances. 4. Results are suggestive that an important action of the veratrum alkaloids may be directly on the vasomotor center. 5. In excessive dosage, germitrine and neogermitrine produce a rise in blood pressure rather than a fall.

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Increase in B-Vitamin Concentrations in Digestive Tract Contents of Young Dairy Calves.* (19280)

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In a previous report(1) the authors presented data which indicated that levels of certain of the B vitamins in the contents of the digestive tract of calves were not related to the age of the animal. Concentrations were as high or higher in calves 2 and 4 weeks of age as in older calves up to 14 weeks, and these levels were greater generally than those in the feed consumed. It seemed desirable to make additional studies using the younger calf in an attempt to determine whether the indicated high values were a consistent phenomenon.

Experimental. Male Holstein calves were quartered and fed in individual pens in an artificially heated and ventilated barn. A medium grade of alfalfa hay and a calf starter were fed *ad libitum* to the animals from the time they entered the barn. Each calf had received colostrum for approximately 3 days, then was placed on a milk feeding schedule which would allow 270 lb of Holstein herd milk during the ensuing 36 days. Twenty calves were divided at random into age groups for slaughter, 5 being sacrificed at each of the following ages: 8, 16, 24 and 32 days. The final feeding of milk occurred 6 hours prior to slaughter; however, hay and grain were accessible to the calf until the time of slaughter. The contents of the rumen, abomasum, small intestine and large intestine were collected in sample jars, iced, taken to the laboratory and blended into a slurry. Chemical assays for thiamine, riboflavin, niacin and pteroylglutamic acid were performed on the slurried contents of the digestive tracts of these calves. Thiamine was determined by the thiochrome method(2), riboflavin by a modification of the fluorometric procedure as set forth by Loy(3), niacin by the cyanogen bromide-metol method(4) and pteroylglutamic acid (PGA) analyses were performed according to the fluorometric method as outlined by Alfrey, *et al.*(5). The milk, grain, and hay which were being fed were similarly assayed several times during the trial. Concentrations of all factors were calculated on a dry matter basis. Since records had been made of the amounts of milk, hay and grain which each calf had received, it was possible to calculate an average level of vitamin intake for each calf on a dry matter basis. This level of intake varied slightly among calves, depending upon the relative proportions of the three feedstuffs consumed.

Results and discussion. The results of the assays for the four vitamins studied are pre-

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TABLE I. Levels of Thiamine, Riboflavin, Niacin and PGA in Digestive Tract Contents of Dairy Calves Compared to Levels in the Feed Consumed.* (μg/g of dry matter).

	Age group (days)	Intake	Rumen	Abomasum	Small intestine	Large intestine
Thiamine	8	5	15.4	2.3	8.3	15
	16	5.1	16.4	4.5	7.9	14.4
	24	5.2	9.7	2.8	6.3	15.3
	32	5.5	10.1	3.9	7.9	12.4
Riboflavin	8	15.8	45.6	13.6	74	256.8
	16	15.3	34.9	13	46.5	153.9
	24	15.3	26.4	14.7	45.3	93.4
	32	14.7	21.6	12.9	42.9	61.7
Niacin	8	7.6	40.9	26	86.6	88.7
	16	7.9	40	8.6	55.5	42.4
	24	9.8	45.2	19.5	103.5	77.4
	32	15.7	36.3	21.8	74.4	39
PGA†	8	7.7	15.6		9.4	55.1
	16‡	6.8	48.4		21.2	66.1
	24	10.6	23.8		26.9	52.4
	32	18	14		31.9	65.3

* Data presented are means for 5 calves slaughtered at each age.
† PGA analyses were run on contents of rumen, small intestine and large intestine only.
‡ PGA data at 16 days on 3 calves only.

sented in Table I, where comparisons are made between levels of each vitamin found in the different regions of the digestive tract and the intake levels in the feed consumed. With the exception of the abomasum, concentrations of thiamine were higher in the material after ingestion than in the feed. Concentrations of this vitamin in the rumen and large intestine were greater than those in the abomasum and small intestine. Levels of thiamine in the samples from the 8-day-old calves were as high or higher than those from the older calves. This lack of difference due to increasing age was noted for all vitamins studied. The results of riboflavin analyses were similar to those for thiamine. Greatly increased levels over those in the feed were found in the rumen, a decrease in the abomasum, then an increase in the small intestine followed by very high concentrations in the large intestine. Much higher levels of niacin were noted throughout the digestive tract than were present in the feed, with the highest concentrations usually in the intestines. PGA determinations were made on the contents of the rumen, the small intestine and the large intestine, the abomasum being omitted. Data presented for the 16-day group are the average of only three calves, the data for the other two being unavailable. With the exception of the 32-day group, concentrations of PGA in the rumen contents were higher than in the feed. The contents of the large intestine exhibited the highest concentrations of this vitamin.

These data show that levels of the vitamins studied were higher in the contents of the digestive tracts of young calves than in the feed. However, no positive proof of microbiological synthesis is offered, since selective absorption of nutrients other than the vitamins studied could result in a concentrating effect of the vitamins in the unabsorbed material in the tract. The differences noted between levels in the digestive contents and the feed are of similar magnitude to those noted by other workers using older ruminants. In addition to whole milk, animals in this experiment had access to hay, grain and bedding. This, plus the fact that they had been with their dams for 3 days after birth, offered opportunity for vitamin synthesizing organisms to develop.

Summary. Concentrations of thiamine, riboflavin, niacin and pteroylglutamic acid were found to be higher in the contents of various regions of the digestive tract of young dairy calves than in the feed consumed. The regions studied were the rumen, abomasum, small intestine and large intestine. Concentra-

tions did not change with increasing age of the calf from 8 to 32 days.

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Development of Tolerance and Cross Tolerance by Mephobarbital.* (19281)

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A review of the literature on the development of tolerance to the hypnotic action of many barbituric acid derivatives in experimental animals has been presented by Gruber and Keyser(1). That cross-tolerance between different barbiturates can also occur has been demonstrated by the same investigators. Since that publication further evidence on the development of tolerance and cross-tolerance between the barbituric acid derivatives has been demonstrated by Carmichael(2), Hubbard and Goldbaum(3) and Holck and his associates(4). There have been reports in the literature, however, which indicate that no tolerance can be developed with mephobarbital (Mebaral)5-ethyl-1-methyl-5-phenylbarbituric acid(5-8) but we have been unable to find any report in which a study has been made to determine actually whether there can or can not be developed tolerance to this chemical in experimental animals. This investigation was therefore undertaken to answer this question and also if tolerance can be developed with mephobarbital in experimental animals does it also produce cross-tolerance.

Methods. Seventeen rabbits, 8 males and 9 females, weighing between 2.1 and 2.6 kg were used. The sodium salt of mephobarbital was employed for injection. This salt was formed by neutralizing the acid with sodium hydroxide and back-titrating with hydrochloric acid to the lowest pH possible without precipitation. Solutions were made up in distilled water and the concentrations adjusted so that 2 cc/kg were given as the final dose. Mephobarbital (40 mg/kg once daily for three days) was injected rapidly, intravenously and the length of induced sleeping time noted following each injection. On the fourth day butabarbital sodium (36 mg/kg) was similarly administered. The same dose of butabarbital was injected on the fourteenth day as a control for this drug. The criterion for development and abolition of sleep was the loss and gain of the righting reflex respectively(9).

Results. Evidence recorded in the literature indicates that there is no essential sex difference in rabbits in response to the action of certain barbiturates(1,9,10). Our results are in accord with these findings. Following the injections of mephobarbital on the first, second and third days, the males slept an average of 19.5, 7.7 and 7.3 minutes respectively while the females slept an average of 16.2, 10.1 and 4.8 minutes respectively. The results with the use of butabarbital also showed no significant difference in the sleeping times between the males and females. Therefore the results obtained from the male and female animals were combined. The mean sleeping times for the first, second and third daily injections of mephobarbital were 17.8, 8.9 and 5.9 minutes respectively and two times the standard errors were $\pm$ 5.4, $\pm$ 1.9

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and ± 1.9 respectively. Thus, in the case of mephobarbital, the sleeping time was reduced 67% in three days, showing a definite development of tolerance. Two times the standard error of the mean (95% confidence limits) were used to indicate the significant difference of the means.

On the fourth day following the three daily injections of mephobarbital a butabarbital sodium injection caused the rabbits to sleep an average of 46.6 minutes. This is well below the normal sleeping time for the dose used(1). The fact that the sleeping time was greatly increased (85.7 minutes) with the same drug and the same dose on the fourteenth day, after a ten day interval without drugs, by which time the tolerance should be lost as was shown by Gruber and Keyser(1), would indicate that cross-tolerance also is produced.

Summary. (1) Repeated daily intravenous injections of mephobarbital produces tolerance in experimental animals (rabbits). (2) The development of tolerance to mephobarbital also produces cross tolerance to butabarbital sodium.

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Post-Irradiation Studies on Mammalian Testes. Effect at Hourly Intervals for First 24 Hours.*† (19282)

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The differential response of the germinal elements in the testes of adult pure strain mice subjected to direct x-irradiation follows a characteristic pattern when examined by quantitative random sampling. This observation is based on a tabulation of the occurrence of a given type of germ cell in 100 observed cross-sections of testicular tubules. The tubules were chosen at random from multiple sections of both testes in order to give fair sampling.

In this investigation the incidence of the various germinal cells at hourly intervals in the 24-hour period post-irradiation was observed. The dose used is known to lower the incidence of germinal cells to a level of 3% (4) by 26 days post-irradiation. The present study is a continuation of the general project of accumulating quantitative data on the biological effects of direct x-irradiation as a contribution to the fundamental problems of normal cell growth and neoplasia. Sensitivity of germinal cells as a result of over-all irradiation, or by the use of radioactive isotopes, has been discussed(1). A review of the literature, with a summary of some of the findings as they apply to the general effect of irradiation on mammalian testicles, was given previously(5). It has long been clear that spermatogonia are the first to show effects following irradiation. The relative sensitivity of other germinal cells, however, needs further investigation. For example, Bloom(1) comments on the relative radioresistance, while

* The authors wish to express their appreciation to Miss Kathleen McCarthy, Pondville Hospital, for cooperation in collection of data and assisting in this project.

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TABLE I. Incidence of Frequency of Germinal Cells in Irradiated Testes. Hourly interval study in 24 hr period post-irradiation.

	Spermatogonia			Spermatocytes	
Time, hr	Resting	Mitotic	Reticulated or prophase-like	Nucleated	Meiotic
1	44	0	42	88	8
2	34	1	38	88	8
3	38	0	45	87	16
4	31	0	48	86	13
5	31	0	43	85	12
6	36	0	47	86	10
7	21	0	44	85	9
8	38	0	47	87	5
9	40	0	48	83	10
10	28	0	41	93	9
11	42	0	39	90	9
12	43	0	44	91	12
13	37	0	38	91	13
14	35	0	41	94	7
15	32	0	44	85	14
16	41	0	41	92	11
17	38	0	43	87	13
18	41	0	47	80	12
19	23	0	50	88	14
20	29	0	45	94	13
21	15	0	33	96	12
22	12	0	26	97	18
23	12	0	35	99	16
24	16	0	38	92	10

Eschenbrenner and Miller(3) suggest that all stages of spermatogenesis may be equally sensitive. Tullis and Barrow(9) note that the basement membrane cells survive longer than the "prespermatogonia" (Bloom) after total-body exposure to 1100 r of x-rays.

Procedure. The technic employed for direct irradiation of the testes has been described in detail(4). It involves direct irradiation of the testes with the rest of the animal shielded. Adult C57 black mice, 50 to 80 days of age were used. Radiation was obtained from a G.E. Maximar, 250 KvP x-ray unit. The following factors were used: 100 KvP, 15 Ma Hvl 2.6 mm Al, 187 r per minute at TSD of 20 cm, for 18 min. 40 sec., total dose 1440 r. All irradiation procedures were carried out from 9 a.m. to 12:00 noon. Two animals were killed at hourly intervals through the 24-hour period post-irradiation (Table I). Untreated animals, 2 for each interval, were killed at hourly intervals for a period of 24 hours. The 2 animals used at each interval were considered adequate for random sampling unless data revealed marked biological variation.‡ Sections representative of the whole of both testes were prepared for microscopic study. The material was fixed in Zenker's solution and stained with Heidenhain's iron haemotoxylin.

Observations and discussion. The tabulation of the frequency of occurrence in untreated animals of resting, mitotic and prophase-like spermatogonia and of the nucleated and meiotic spermatocytes was calculated. The results may be summarized as follows: at any interval approximately 34 tubules out of any 100 observed will reveal the presence of

‡ In determining whether the sample used in this experiment was large enough, trends were computed from the data and tested for significance. Two methods were used in computing trends: (1) Trends were constructed for both normal and irradiated cells, using averages of the 2 figures in a sample; (2) Two individual trends were constructed for each sample of the irradiated group, and the 2 individual probabilities were multiplied together. No significant trends were found in the control group. It would appear that with differences as great as those found in the irradiated group, the number of mice used is significant. The only overall significant downward trend is in the irradiated resting spermatogonia. (Report by Dr. Herbert L. Lombard, Massachusetts Division of Cancer and Other Chronic Diseases, Boston, Mass.)

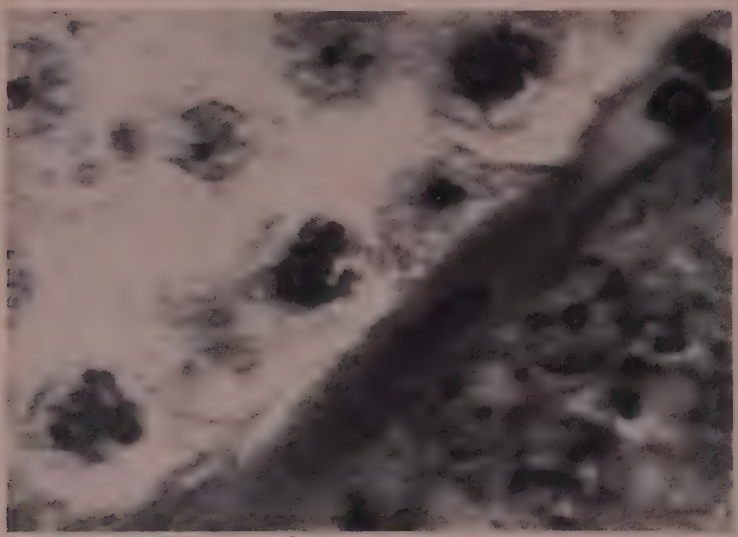

FIG. 1. Normal untreated testis from C-57 pure-lined black mouse. Zenker fixation. Section—6/¹. Heidenhain's hematoxylin. The tubule on the left shows 2 typical resting spermatogonia. These can be seen at the periphery. The tubule on the right shows the prophase-like cells lining the periphery.

resting spermatogonia, mitotic spermatogonia will appear 2-3 times out of 100, prophase-like spermatogonia 41 times out of 100, nucleated spermatocytes 86 times and meiotic spermatocytes 10 times.

Spermatogonia. A typical resting spermatogonium, observed in C57 black mice, is seen in Fig. 1. The nucleus is characterized by a definite nuclear membrane, several nucleoli, and dust-like chromatin scattered on a delicate fibrillar network. This is suggestive of the "dust-like" nucleus described by Bloom(2) and also noted by Tullis and Barrow(9). Location of this cell is limited to the periphery of the tubule. The other predominant spermatogonial cell is also represented in Fig. 1. It is characterized by apparent prophasic chromatin threads and may or may not have a nucleolus. Preliminary observations cast some doubt on the identity of this cell. It is possible that it may either proceed into active mitosis and produce two new spermatogonia, or increase in size, develop a prominent spermatocytic nucleolus and evolve into a spermatocyte. Further cytological studies of this cell are in progress. In the examination of 100 cross-sections of tubules of normal testes, spermatogonial mitosis is found to be infrequent. When it does occur there may be several mitotic figures in one median cross-section of a tubule. It is indicated, for example, that of 100 cross-sections examined, only one tubule revealed mitotic spermatogonial cells. This emphasizes the need for investigation of the regenerative rate of the spermatogonial cells. No attempt has been made to differentiate between the various types of resting spermatogonia that have been described in the literature, such as, for example, the large and small cells, or the spermatogonia and the spermatogonia of Bloom(1), nor to differentiate between the variations in intensity of stain.

Spermatocytes. In assembling data on spermatocytes, only two types were considered: (1) the spermatocyte in any phase where the nuclear wall was still present and (2) the active meiosis, whether late prophase, metaphase or telophase. No attempt was made to distinguish between primary and secondary spermatocytes. The nucleated spermatocytes showed little variation in incidence at any interval during the 24-hour period. The meiotic spermatocytes showed some variation but were more or less in a given range for frequency. It is interesting

to note that the incidence for meiotic spermatocytes was higher than for mitotic spermatogonia.

Post-Irradiation. The effects at hourly intervals for 24-hours after acute irradiation are summarized in Table I. It is apparent that (Table I): (a) Spermatogonial mitoses disappear. (b) The resting spermatogonia maintain normal incidence for 20 hours. By 24 hours there is a marked decrease. (c) Prophase-like cells, resting spermatocytes and meiotic spermatocytes appear as frequently as in the controls. These data are in agreement with the general belief that, of the male germ cells, the spermatogonia are the first to react to irradiation. They give further statistical confirmation to the observation that dividing spermatocytes "are on the whole quite radioresistant at least for the first 24 hours" Bloom (1). The reticulated or prophase-like cells in this study do not appear as sensitive as either the mitotic spermatogonia or the resting spermatogonia in the later intervals of the 24-hour period. This is in seeming contradiction to the general belief that cells in prophase are the most sensitive to irradiation (Henshaw)(6). The reticulated or prophase-like spermatogonium may, therefore, represent a differentiated stage, not a typical prophase. Disappearance of cells post-irradiation can be due to progression through differentiation to another type of cell or to degeneration. Therefore, some preliminary observations have been made on cells which are apparently in process of degeneration and on cells which show abnormalities.

Such observations should contribute to earlier evidences of biological effects of radiation than was possible by the presence-or-absence tabulations. Abnormal cells are considered to be those which have fragmented chromosomes, multipolar spindles and variation in size, shape and staining capacity. It is possible that these abnormal cells will recover. Degenerating germinal cells are those which are patently undergoing destruction. The criteria for degeneration are pyknosis, eosinophilic cytoplasm, giant cell formation or cellular disorganization.

The occurrence of degenerating cells is recorded in Table II. In some fields both normal and degenerating forms of any one type of germinal cell are present, but the data are kept separate to avoid confusion.

TABLE II. Incidence of Frequency of Degenerating Germinal Cells in Irradiated Testes. Hourly interval study in 24 hr period post-irradiation.

	Spermatogonia			Spermatocytes	
Time, hr	Resting	Mitotic	Reticulated or prophase-like	Nucleated	Meiotic
1	4	0	3	7	0
2	10	0	3	5	0
3	2	0	15	2	0
4	1	0	7	3	1
5	4	0	4	3	1
6	5	0	10	5	3
7	7	1	22	7	2
8	17	1	28	7	2
9	9	0	31	10	0
10	13	0	20	7	3
11	18	0	14	4	2
12	29	0	35	30	9
13	24	0	29	23	6
14	24	0	33	10	6
15	22	0	17	6	9
16	23	0	11	4	7
17	20	0	11	3	10
18	19	0	7	5	11
19	13	0	10	7	10
20	14	0	14	13	11
21	3	0	5	7	9
22	3	0	6	20	15
23	4	0	7	14	13
24	2	0	8	7	4

The frequency of degenerating spermatogonia increased notably in the period from 8 to 20 hours. Emphasis is attached to the observation that a decline in incidence of resting spermatogonia follows the 20-hour interval. This suggests that some of the spermatogonia are actually undergoing degeneration and not proceeding into spermatocytes. The reticulated or prophase-like spermatogonia also show increased degeneration 6 to 20 hours after irradiation but without the consequent effect noted above for resting spermatogonia. This suggests that these spermatogonia-like cells are reacting to irradiation more like spermatocytes than spermatogonia.

In the 24-hour interval there was no lowering of incidence of spermatocytes although evidences of degeneration were present.

It is recognized that degeneration does occur in the testes of untreated animals. The frequency appears to be of the order of 1 to 6 in 100 tubules. These degenerating cells are few in number in any given tubule.

Summary. Quantitative data are presented to show incidence of spermatogonia and spermatocytes in normal mouse testicles at stated intervals in a 24-hour period, and compared with data for testicles of mice killed at hourly intervals the 24 hours post-irradiation. Testicles were exposed to a single dose of 1440 r for 18 min. 40 sec.

The data indicate that within the 24 hours a pattern of response can be demonstrated which is limited to the spermatogonia. Active mitosis is inhibited for at least the first 24 hours and resting spermatogonia decrease in incidence after 20 hours. Reticulated spermatogonial cells, resting and meiotic spermatocytes do not decrease during the interval studied. Supplementary effects of irradiation are noted by a tabulation of the frequency of degenerating cells, confirming the belief that mitotic spermatogonia and resting spermatogonia are the most sensitive germinal cells. Degeneration of the reticulated spermatogonial cells does not result in a lowered incidence of these cells at any time in the 24-hour period.

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Effect of Methylcellulose on Growth Response of Rats to Low Vitamin Intakes. (19283)

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With increasing use of methylcellulose as a laxative in the treatment of chronic constipation(1-4), the question naturally arises—will continued ingestion significantly decrease the intestinal absorption of certain essential nutrients? Perhaps the question has already been answered in the negative by the fact that no clinical symptoms of vitamin deficiencies have been reported in patients who have ingested methylcellulose for long periods of time. Musick(1) cites the unpublished work of Shapiro, who showed that daily ingestion of methylcellulose did not interfere with the absorption of vit. K. However, for more

direct evidence we have studied the effect of ingestion of methylcellulose on the growth response of normal and depleted animals to minimal dosages of thiamine or vit. A. Thiamine and vit. A were chosen as representative of the water-soluble and oil-soluble vitamins, respectively.

Experimental. The given vitamin was dissolved or dispersed in a solution of methylcellulose of such concentration that daily administration by stomach tube of 2.0 cc supplied to each rat the desired quantities of the vitamin and methylcellulose. For the groups receiving no methylcellulose the daily dose of the vitamin was dissolved or suspended in 2.0 cc of water, if water-soluble, or in 0.5 cc of cottonseed oil, if oil-soluble. All dilutions were freshly prepared each day from assayed stock preparations of the vitamins. The experiments were of 28-day duration, during which time the weight responses of the experimental animals were observed. Each group consisted of 5 male and 5 female rats. In all instances, when methylcellulose was administered, a dosage of 50 mg per rat was employed. *Thiamine.* Both normal stock rats and thiamine-depleted animals were used. The depletion period was 18 days. The rats were on a thiamine-free diet* and were fed 6 μg of thiamine hydrochloride daily by stomach tube, with or without simultaneous administration of methylcellulose.† The thiamine was supplied as either crystalline vit. B_1 or thiamine in admixture with other vitamins.‡ *Vit. A.* Stock rats were depleted of vit. A according to the procedure in the U. S. Pharmacopoeia XIV, page 789. Each deficient rat was given 3 units of vit. A daily as either an aqueous vitamin preparation‡ or a fish liver oil concentrate,§ with or without simultaneous administration of methylcellulose.

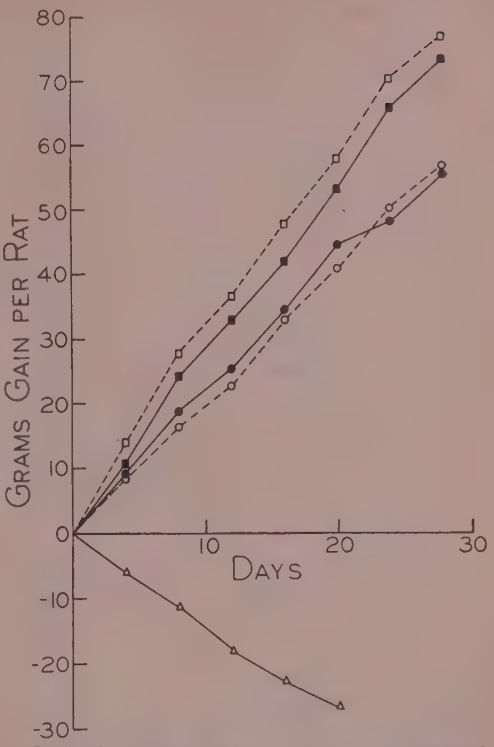

FIG. 1. Weight response curves of B_1-depleted rats given 6 μg of thiamine daily for 28 days with and without 50 mg of methylcellulose. Legend: △——△ control, ●——● cryst. B_1 and methylcellulose, ○---○ cryst. B_1, ■——■ B_1 as aq. vit. preparation and methylcellulose, and □---□ B_1 as aq. vit. preparation.

Results. In Fig. 1 and 2 are plotted the growth response curves of the various groups of animals. Since the response of the groups of normal rats was almost identical with that of the groups of thiamine-depleted rats, curves for the latter animals only are plotted in Fig. 1. Examination of the figures reveals that daily ingestion of methylcellulose by the rat does not significantly change the growth response to minimal doses of vit. A or B_1.

Discussion. Dutcher and coworkers(5) demonstrated, in the rat, that administration of mineral oil with varying amounts of carotene decreases the intestinal absorption of

* Thiamine-free diet: 18% casein (vit.-free), 4% salt mixture, 8% cottonseed oil, 5% autoclaved yeast, 2.5% Ruffex, 60.5% starch, 1% wheat germ oil and 1% cod liver oil.

† Dow Chemical Co. Methocel, 400 cps., Pharmaceutical grade.

‡ Known under the trade name of Poly-Vi-Sol. It contains, in addition to vit. A palmitate, irradiated ergosterol, ascorbic acid, thiamine hydrochloride, riboflavin, niacinamide and small amounts of inert ingredients.

§ Known under the trade name of Mead's Oleum Percomorphum. Consists of liver oils of percomorph fishes, Viosterol and other fish liver oils.

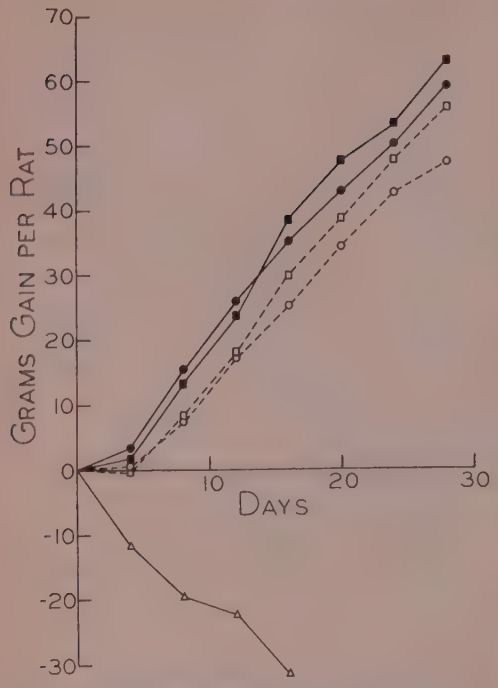

FIG. 2. Weight response curves of A-depleted rats given 3 units of vit. A daily for 28 days with and without 50 mg of methylcellulose. Legend: △——△ control, ●——● A as aq. vit. preparation and methylcellulose, ○- - -○ A as aq. vit. preparation, ■——■ A as fish oil and methylcellulose, and □- - -□ A as fish oil.

carotene, but that mineral oil has little effect on the absorption of vit. A administered in cod liver oil or as a vit. A concentrate. The explanation proposed for the different effects of mineral oil was the greater solubility of carotene in mineral oil than in the intestinal fluids and the greater solubility of vit. A in the intestinal secretions than in mineral oil. More recently, however, Paul, Ellis, and Paul (6) propose that the decreased effectiveness of β-carotene administered in mineral oil may be the result of partial isomerization of active β-carotene to stereoisomers of lower biological activity and that stereoisomerization of vit. A in mineral oil does not result in decreased biological activity. Whatever the true explanation may be for the effect of mineral oil on the utilization of carotene and of vit. A by the rat, these studies emphasize the possibility that ingestion of methylcellulose might decrease the effectiveness of the oil-soluble vitamins in the animal.

In order to learn whether ingestion of methylcellulose might decrease absorption of water-soluble or oil-soluble vitamins, low dosages of vit B_1 or A were fed to normal stock animals or depleted rats, and the growth responses were observed. Earlier work with vit. A(7) indicates that if decreased absorption were significant it would be reflected in decreased growth of the rats. The dosage of methylcellulose employed was 0.7 to 1.0 g per kg body weight which is approximately equivalent to 10 times the dosage ordinarily given to man.

From the weight gain of the thiamine-depleted and normal animals given 6 μg of thiamine daily for 28 days with and without simultaneous administration of methylcellulose, it is evident that the latter agent does not reduce the effectiveness of the low thiamine dosage and, therefore, does not decrease the intestinal absorption of the water-soluble vitamin. Similarly, the weight gains of the A-depleted rats show that the simultaneous administration of methylcellulose with 3 units of vit. A does not decrease absorption of the oil-soluble vitamin.

Conclusion. Daily ingestion of methylcellulose does not significantly decrease the effectiveness of (1) thiamine hydrochloride in the depleted or normal rat, or (2) vit. A in the A-depleted rat. These data are interpreted to mean that daily ingestion by the rat of large amounts of methylcellulose does not decrease the absorption of water-soluble or oil-soluble vitamins, and therefore does not increase the daily requirement of the rat for these factors.

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Effect of Choline on Yeast Bioassay of Inositol.* (19284)

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During the application of the inositol microbiological assay of Atkin, Williams, Schultz, and Frey(1) to the determination of lipid inositol in blood plasma, it was noted that inhibition of the yeast growth occurred in the presence of plasma hydrolysates. This inhibition occurred with completely hydrolyzed lipid samples, insofar as all the lipid P was recovered as inorganic phosphate and was partially overcome with increasing amounts of the hydrolysate. It was found further that the free choline present was solely responsible for this effect. In this paper are summarized the effects of different amounts of choline and several related substances on yeast growth with standard amounts of inositol.

Methods and results. The basal medium was prepared according to the authors' method as reported by György(1), except that a commercial casein hydrolysate was used (Nutritional Biochemicals Corp.) and the pH of the medium was adjusted to 4.38. The assay was carried out in 25 ml Erlenmeyer flasks in a 30°C water bath for 16 hours. Growth was read turbidimetrically in a Coleman Universal Spectrophotometer at 600 μ. Identification of the inhibiting agent in plasma hydrolysates was ascertained after a check assay of a synthetic "hydrolysate," containing 21 $\mu\gamma$ M of inorganic phosphorus, 33 glycerol, 20.9 choline, 1.16 ethanolamine, 2.32 dl-serine, 60 μg inositol. With this mixture, typical inhibition occurred. It was found that choline alone was responsible for this inhibition. The other substances did not inhibit at the above levels or at levels equivalent to 31 times the inositol present on a molar basis.

Other compounds related chemically or metabolically to choline were also tested with inositol at molar ratios of 30 or 60 to 1. It was found that dimethyl ethanolamine inhibited growth of the yeast to the same extent as choline, whereas methionine, betaine, and choline phosphatides from liver had no effect.

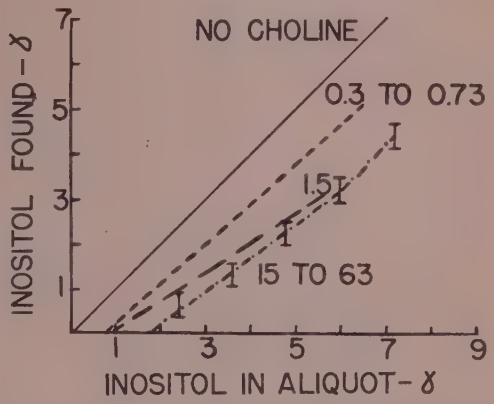

FIG. 1. Effect of choline on inositol assay using *S. carlsbergensis*. The figures accompanying the above curves represent the choline to inositol molar ratios of the particular samples assayed.

Variations in the choline content of hydrolysates of lipid extracts made it necessary to determine the effect of choline at various levels. Solutions were assayed containing choline to inositol molar ratios of 0.3 to 63. These results are given in Fig. 1. It is apparent that molar ratios as low as 0.3 cause measurable inhibition whereas maximum inhibition is reached at a ratio of 15:1 and possibly lower. Therefore, it is possible to establish a standard curve with choline present in excess of a 15:1 molar ratio and obtain values for plasma hydrolysates directly from this curve. A comparison of values obtained from such a curve with those read from the regular, "non-choline" curve has been made and is recorded in Table I. It appears that results calculated from the "choline" curve in this manner are more consistent and essentially valid.

Discussion. There is little information available on the effect of tissue preparations or biochemical substances on the growth of *Saccharomyces carlsbergensis* using this assay. Williams *et al.*(2) have reported that extracts of many biological materials contain sufficient amounts of stimulatory or inhibiting sub-

* This work was supported by a grant from the Life Insurance Medical Research Fund.

TABLE I. Comparison of Plasma Lipid Inositol Values Based on Standards With and Without Added Choline.

Plasma hydrolysate dog sample	Inositol found—calculated from normal standard curve		Inositol found—calculated from standard curve with added choline	
	Range γ moles	Avg γ moles	Range γ moles	Avg γ moles
A, B	4.49–6.64	5.47	10.7 –12.17	11.46
C	1.01–2.41	1.97	3.70– 3.99	3.89

stances to interfere with the inositol assay using *S. cerevisae*. These substances were not identified and their effects were blanked out by addition of an autolysate of the material to be tested. In the basal medium for *S. cerevisae* devised by Woolley(3,4) the choline to inositol molar ratio at the assay level is approximately 5:1 on the basis of added choline alone. Hence if *S. cerevisae* is inhibited by choline similarly to *S. carlsbergensis* this effect would largely be obscured by the choline content of the basal medium. On the other hand, the choline effect may be specific for the *S. carlsbergensis*.

The low molar ratios of choline to inositol capable of inhibiting the growth of *S. carlsbergensis* limit the use of this assay under these conditions. Many tissues or tissue extracts would be expected to contain choline equivalent to a choline to inositol molar ratio of at least .3. However, this effect can be readily corrected for by the addition of sufficient quantities of choline to the basal medium.

It should be emphasized that the capacity of the organism to overcome the inhibition is enhanced with higher levels of inositol for the amount of inhibitor is also proportionately increased. Inositol then appears to be necessary for the removal of choline. The failure of betaine, ethanolamine, and choline phosphatides to inhibit suggests metabolic pathways by which the inositol fed yeast might reduce the choline content of the medium. It is possible that one or more enzymes concerned with the conversion of choline to these substances may require inositol for its synthesis or action.

Summary. Growth inhibition of *S. carlsbergensis*, caused by choline during routine inositol assays has been described. The effect was noted at a choline to inositol molar concentration of 0.3 to 1 and maximum inhibition was reached at a ratio of 15:1. Similar inhibition occurred with dimethylamino ethanol but was not observed with ethanolamine, serine, betaine, methionine or choline phospholipides. For assay purposes, this inhibition was corrected for by the addition of comparable amounts of choline to the standard inositol tubes.

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Studies on Renal Excretion of Beryllium.* (19285)

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Considerable attention has been given in the recent literature to the problem of beryllium poisoning(1-6). The distribution and excretion of beryllium have been studied by several workers(7,8). In pursuing further knowledge regarding the interactions of beryllium in the body, it was of interest to study the mechanism by which this metal is handled by the kidney. By combining renal clearance measurements with plasma ultrafiltration studies, it was hoped to show whether beryllium is excreted by glomerular filtration or by means of tubular activity. While this work is by no means definitive, a strong suggestion has been obtained that the latter must play an important role.

Experimental. While the rabbit is not usually the animal of choice for renal clearance studies, rabbits rather than dogs were employed in the present work because of the high cost of the Be^7 isotope used. Although clearance values often vary widely from one rabbit to another and are extremely sensitive to differences in the rate of urine flow, the use of clearance ratios, where the clearance of the substance being studied is referred to the simultaneous clearance of a standard substance such as inulin, minimizes such difficulties(9). Young male rabbits were fasted for about 18-20 hours preceding the beginning of the experiments. About 2 hours before the experiments, water (30 ml/kg) was administered by stomach tube; this was repeated 3 times at half-hourly intervals. About 1 hour before the experiments, inulin (10 ml of a 20% solution in isotonic saline) was given subcutaneously, followed by a similar intravenous dose about 30 minutes before the experiments. Just before the start of the experiments, the rabbits were tied down on a rabbit board and catheterized. The Be^7 solution, containing about 5 mg of citric acid to keep the beryllium in soluble form before injection, and adjusted to physiological pH, was given intravenously. A few minutes after the isotope injection, the bladder was washed repeatedly with warm water, and the collection of urine was begun. Urine was collected for about 30 minutes, after which the bladder was washed repeatedly and the washings added to the urine sample. In most of the experiments, 3 such collection periods were run. Blood samples (about 2 ml) were obtained from the peripheral vein of the ear at 10-15 minute intervals throughout the urine collection periods. The blood was collected in heparinized tubes and centrifuged immediately. The plasma was removed, and together with the urine samples stored in the refrigerator prior to analysis. In several of the experiments, an additional blood sample of about 10-15 ml was withdrawn for the purpose of determining the ultrafiltrability of the beryllium. This sample was taken early in the experiment so that the Be^7 activity would be fairly high. The ultrafiltration was carried out immediately after collecting the blood sample. The latter was centrifuged, the plasma activity measured, and ultrafiltration was carried out using a centrifuge type of apparatus essentially the same as that described by Feldman *et al.*(10). These authors had studied the permeability of the cellophane membrane employed: it was found to be permeable to small colloidal particles such as inulin, but impermeable to beryllium hydroxide and protein; beryllium in 0.001 M hydrochloric acid readily passed through the membrane, as did beryllium in neutral solution in the presence of complexers such as citrate. Plasma and urine samples were counted with a Geiger-Muller counter equipped with a dipping tube. In all cases, sufficient counts were taken to give a counting error of less

* This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project.

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than 3%. Inulin determinations were carried out by Harrison's modification of the diphenylamine method (11). In tests of the method on analyses of plasma samples containing known quantities of inulin in concentrations similar to those encountered in this study, errors greater than 5% were never found.

Results. The results of all the clearance determinations are summarized in the accompanying table. It may be seen that in those cases where citrate was not added to insure the solubility of the beryllium prior to injection, the beryllium clearance was very small as contrasted with those cases where soluble beryllium was injected. This might suggest that the beryllium, once in an insoluble form, is resistant to the action of physiological complexers to the extent that it never, during the course of the experiments, assumed the same chemical form as did soluble beryllium. It is strange to note, however, as pointed out below, that even "soluble" beryllium becomes non-diffusible after equilibration with plasma. Further studies regarding the state of beryllium in the plasma will be required for an explanation of these findings.

A second observation of interest is that the addition of carrier did not markedly alter the beryllium clearance. It may be pointed out that while only 180 γ of carrier was added, this represents an increase of several orders of magnitude over the quantity of beryllium in carrier-free Be^7.

Third, it may be seen that there is little correlation between the inulin clearance and the beryllium clearance. The clearance ratio varies sufficiently widely, not only from one rabbit to another but also with the same rabbit in different urine collection periods, to suggest that beryllium is excreted by a mechanism different to some extent from that involved in inulin excretion. Errors in the methods used could account for only a small part of the variation. This suggests tentatively the idea of tubular activity in beryllium excretion.

The results of the plasma ultrafiltration study support this view. These results may be summarized by stating that in no case was any part of the plasma beryllium found to be ultrafiltrable. Plasma samples containing several hundred counts per minute per ml invariably yielded filtrates contain-

TABLE I. Summary of Clearance Data.

Rabbit No.	Description of dose	Inulin clearance (ml/min)	Beryllium clearance (ml/min)	Clearance ratio, C_{Be}/C_{inulin}
1	Carrier-free Be^7 without citrate*	6.6	1.8	.27
		8.7	1.8	.21
		7.8	1.6	.21
		8.2	3.5	.43
2	Same	12.1	.82	.07
		15.5	1.2	.08
		4.7	.26	.06
		1.3	0	0
3	Be^7 with 180 γ carrier and citrate†	11.6	6.6	.57
		14.2	14.1	.99
4	Carrier-free Be^7 with citrate†	7.6	7.1	.93
		14.9	22.3	1.5
		11.7	7.9	.68
5	Same	19.8	14.6	.74
		14.9	8.1	.54
		9.2	4.1	.45
6	Same and PAH and carinamide‡	8.3	6.8	.82
		11.4	8.1	.71
		2.6	2.8	1.1

* Citrate was not added to insure solubility of the beryllium before injection.

† Citrate, a potent complexer of beryllium, was added to the solution before the pH was raised to physiological value prior to injection; thus the beryllium was administered in the form of a true solution.

‡ In an attempt to demonstrate tubular secretion of beryllium, this rabbit was given 1 g of carinamide by stomach tube 1 hr before the experiment, and 1 g of p-aminohippuric acid intravenously repeated at 3 half-hourly intervals during the experiment.

ing no more than 5 or 10 counts per minute per ml, in other words, not significantly greater than background. In view of the studies of Feldman *et al.*(10) using the same type of apparatus, it is certain that the failure to find beryllium in the ultrafiltrate represents a true non-diffusibility and is not an artifact resulting from the apparatus employed. While it is not considered that the cellophane ultrafilter resembles in all regards the glomerular filtration mechanism of the kidney, such *in vitro* studies often lead to significant suggestions. Taken together with the clearance data, the ultrafiltration studies point toward the involvement of tubular activity in beryllium excretion. The clearance of a non-filtrable substance seems unusual, but there is insufficient data to discuss it further. Studies on the state of beryllium in the blood, now in progress, and further work on the renal mechanisms involved, will be required for elucidation of the problem.

As shown in the table, an attempt to demonstrate an inhibitory effect of p-aminohippuric acid and carinamide on possible tubular excretion of beryllium was not successful, but because of the variety of tubular secretory systems, negative findings in such an experiment prove little.

Summary. Carrier-free Be^7 injected in an insoluble form has a very low clearance as compared with the simultaneous inulin clearance. When citrate, a potent complexer of beryllium, is added to insure that the injected beryllium is in an insoluble form, the clearance is much greater, despite the fact that ultrafiltration studies indicate the soluble beryllium to assume a non-diffusible form after mixing with the blood. The addition of carrier does not markedly alter the beryllium clearance. The clearance ratio, *i.e.*, C_{Be}/C_{inulin}, varies widely enough to suggest that the two substances are not excreted by the same mechanism, and the possibility of tubular activity in beryllium excretion comes to mind. This idea is supported by ultrafiltration studies which show that beryllium exists in the plasma in a non-diffusible form. Complete clarification of the problem must await further work on the state of beryllium in the blood.

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Differential Rates of Diffusion of Mannitol from Phases of Extracellular Compartment in Edematous States.*† (19286)

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The development of a simple and accurate

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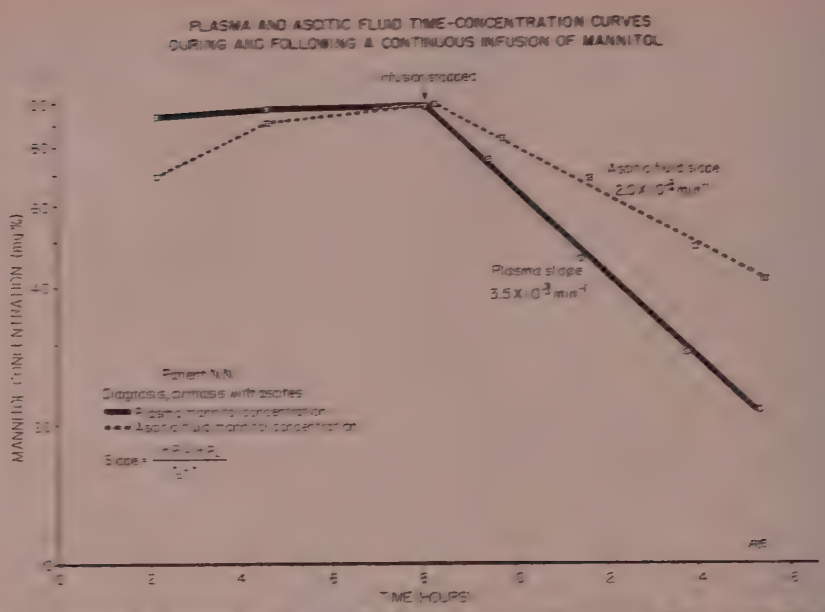

FIG. 1. Example of plasma and ascitic fluid time-concentration curves during and following a continuous infusion of mannitol.

method for measuring extracellular fluid volume has been the subject of much investigation. Recently a series of three carbohydrates, sucrose(1,2), mannitol(3-5), and inulin(6,7) have been used to estimate extracellular volumes. These substances obviate the disadvantage of variable entrance into cells, diffuse into a smaller space of distribution and presumably more accurately estimate the extracellular volume(8). A procedure for determining the mannitol space has been reported that was independent of route of removal of mannitol and corrected automatically for any extrarenal disposal(9). The procedure (infusion-slope method) has the added advantage in that it obviates urine collections. Close correspondence has been observed between the inulin space as measured by the infusion-recovery method and the mannitol space measured by the infusion-slope method (10). These data would seem to establish the validity of both methods for measuring the extracellular fluid volume. It should be noted that these studies were carried out in normal subjects.

An important requirement in the determination of the mannitol space by the infusion-slope method is that the clearance rate of mannitol from plasma water and from all other recesses of the extracellular phase be comparable after the mannitol infusion is stopped. The purpose of this study was to test the validity of this assumption by simultaneously measuring the comparative rates of diffusion of mannitol from plasma and ascitic fluid in patients with cirrhosis and from plasma and peripheral edema fluid in patients with congestive heart failure.

Methods. Three groups of male subjects were employed, 1) normal individuals admitted for minor surgery, 2) cases of cirrhosis with ascites, and 3) patients with congestive heart failure and peripheral edema. The subjects were allowed food and water *ad libitum* but were kept recumbent in bed throughout the experimental procedure. No preliminary hydration was employed. A priming injection of mannitol was followed by a constant sustaining infusion for 8 hours. Mannitol plasma levels were maintained between 100 and 200 mg %. All solutions were made up in 0.45% saline solution. A Bowman constant injection pump was used for the intravenous infusion at a rate of 1.9 cc/min. with a mean variation of 3.6 ± 2.5% in the volume flow per minute. Ascitic fluid was obtained by

means of an indwelling nylon catheter introduced into the peritoneal cavity through a 17 gauge needle(11). In patients with congestive heart failure, peripheral edema fluid was obtained by the subcutaneous insertion of a special 16 gauge needle prepared with additional holes along the barrel. Blood and interstitial fluid samples were drawn simultaneously at 2, 4, and 8 hours during the infusion and at hourly intervals for 3 hours after the infusion was stopped. Mannitol was determined by the method of Corcoran and Page(12). Mannitol recovery from plasma averaged 100.1 ± 6.5%. Plasma and edema fluid blanks were run routinely. Plasma concentrations of mannitol were corrected for plasma water content according to the concentration of protein present.

Results. When the mannitol infusion was stopped, the concentration of mannitol in body fluids fell rapidly as mannitol was excreted. The logarithms of mannitol concentrations plotted against time proved to be a linear function. (Fig. 1). Taking any 2 points on the regression line, one can calculate the slope as follows:

$$S = \frac{l_n P_1 - l_n P_2}{t_2 - t_1}$$

where S = slope (min^{-1}), P_1 = a regression plasma concentration at t_1, P_2 = a regression plasma concentration at t_2. The magnitude of the slope value is a direct measure of the rate of removal or clearance of mannitol.

Table I summarizes the slope values for mannitol obtained in normal subjects, cases of cirrhosis, and patients with congestive heart failure. The mean plasma slope in normal subjects was 5.0 ± 1.13 × 10^{-3} min^{-1}. This was significantly higher than the mean plasma slope obtained in cirrhotic patients 3.5 ± 0.82 × 10^{-3} min^{-1} P = .05) and that obtained in patients with congestive heart failure 2.7 ± 0.67 × 10^{-3} min^{-1} (P < .01).

From Fig. 1 it can be seen that mannitol diffused more rapidly from the circulating plasma than from ascitic fluid. A summary of differential rates of mannitol diffusion from various recesses of the extracellular compartment is shown in Table I. When analyzed by the method of paired comparisons, the mean differences between plasma and ascitic fluid

TABLE I. Comparative Rates of Diffusion of Mannitol from Various Phases of the Extracellular Compartment.

Normal subjects: Subject	Plasma slopes (1×10^{-2} min^{-1})	Cases of cirrhosis with ascites: Time-concentration slopes (1×10^{-3} min^{-1}) Patient	Plasma	Ascitic fluid	Diff.*	% diff.†	Cases of congestive failure with peripheral edema: Time-concentration slopes (1×10^{-3} min^{-1}) Patient	Plasma	Edema fluid	Diff.*	% diff.†
1. J.M.	3.5	1. J.S.	2.5	2	.5	20	1. A.L.	3.5	0	3.5	100
2. R.N.	5	2. V.G.	4.6	4	.6	13.1	2. G.F.	2	.4	1.6	80
3. K.G.	4.9	3. N.N.	3.5	2	1.5	42.8	3. R.O.	3	.6	2.4	80
4. V.S.	5	4. J.K.	4	2	2	50	4. P.G.	2	.7	1.3	65
5. H.B.	6.7	5. C.R.	3	1.2	1.8	60	5. A.A.	3	2.5	.5	16.7
Mean	5		3.5	2.2	1.3	37.2	—	2.7	.8	1.9	68.3
Stand. dev.	1.13		.82	1.04	.69	19.9	—	.67	.97	1.14	31.4
—	—	P	.05		.03	—	P	<.01	—	.04	—

* Diff. = Plasma slope − ascitic fluid slope.

† % diff. = $\frac{\text{Diff.}}{\text{Plasma slope}}$ / 100.

slopes (1.3×19^{-3} min^{-1}) and between plasma and edema fluid slopes (1.9×10^{-3} min^{-1}) were both statistically significant.

Discussion. The impairment of daytime glomerular filtration rates in this series of cases of cirrhosis and congestive heart failure was evident in the decreased rate of plasma clearance of mannitol. In the measurement of the mannitol space by the infusion-slope method, a basic requirement is that mannitol be uniformly cleared from all recesses of the extracellular compartment. The differences in slope values between plasma vs. ascitic fluid, and plasma vs. interstitial edema fluid, indicated that this requirement was not met in the edematous states studied.

It was of particular interest to note that the mannitol was cleared at a more rapid rate from ascitic fluid than from peripheral edema fluid. Other data indicate that mannitol and inulin equilibrate at a faster rate in ascitic fluid than in peripheral edema fluid(13). This conflicts with any *a priori* concept that ascitic fluid is relatively adynamic in contrast to peripheral edema fluid, and is in agreement with recent findings on the rapid exchange of labelled protein in experimental ascites(14).

Conclusions. 1. The plasma clearance of mannitol observed during morning hours was significantly decreased in this series of cases of cirrhosis with ascites and congestive heart failure with edema. 2. Rates of diffusion of mannitol from various recesses of the extracellular compartment were not uniform in two edematous states, namely, cirrhosis with ascites and congestive heart failure with edema. 3. The mannitol method as determined by the infusion-slope procedure cannot be applied to the measurement of the extracellular fluid compartment in certain edematous states.

The authors are indebted to Dr. Smith Freeman for his helpful criticisms in the planning and execution of these studies. The authors also wish to gratefully acknowledge the support and unfailing interest of Dr. Charles B. Puestow, Chief of the Surgical Services and Dr. Lyla A. Baker, Chief of the Medical Services at Hines Hospital.

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Plasma Protein. IV. Results with Generally Labeled Plasma Protein.* (19287)

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We have reported(1,2) on the metabolism of plasma protein labeled by feeding donor rats serine-β-C^{14} and administering the C^{14}-

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labeled protein to recipients. In these experiments it was found that the C^{14}-labeled protein rapidly disappeared from the blood circulation when intravenously injected, and rapidly appeared in the blood circulation when intraperitoneally injected. Metabolism of the protein occurred at such a rate as to give a half life to 3.1 days. The maximum rate of $C^{14}O_2$ production was attained in 2 to 4 hr whether the labeled protein was given intravenously or intraperitoneally. After oral administration of the labeled protein the rate of $C^{14}O_2$ production reached its maximum still earlier in the experiment but its activity fell rather rapidly. The C^{14} also appeared in the tissues, very rapidly in some such as liver, more slowly in others such as muscle.

It was thought advisable to check these results using a different method of labeling the proteins in the donor animals. To this end we have fed the donors killed C^{14}-labeled *Rhodospirillum rubrum* organisms. In these organisms all the amino acids were labeled though perhaps not uniformly. Data concerning their preparation will be given in another communication.‡ The results show that the rate of loss of these two types of labeled protein from the circulation is the same but that there are differences in the rate of appearance of the C^{14} in the respiratory carbon dioxide and in the tissues.

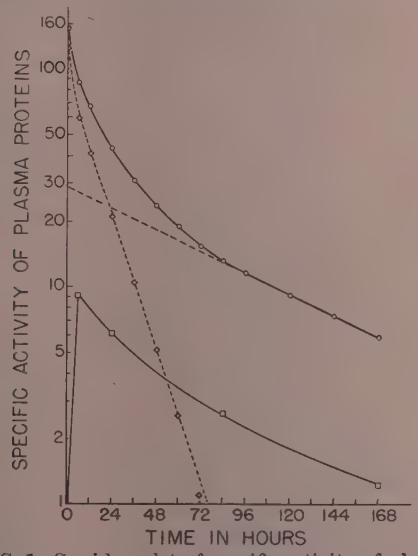

FIG. 1. Semi-log plot of specific activity of plasma protein of Recipients B. Upper unbroken curve (circles) represents average values obtained from 4 rats given plasma protein intravenously and 4 rats given plasma protein intraperitoneally (after 6 hr in the latter); lower unbroken curve (squares) values from 5 rats given plasma protein orally. Regarding broken curves see text.

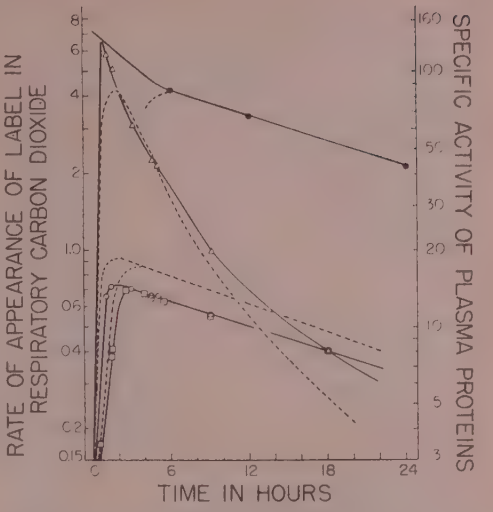

FIG. 2. (a) Semi-log plot of specific activity of plasma protein (of Recipients A or B) vs. time (solid circles). (b) Semi-log plot of rate of appearance of C^{14} in the respiratory CO_2 of Recipients B, expressed as % of dose per hr vs. time (open circles, after intravenous injection; squares, after intraperitoneal injection; triangles, after oral administration). (c) Dotted curves, semi-log plot of rate of appearance of C^{14} in the respiratory CO_2 of Recipients A expressed as above (same curves as Fig. 2, paper I).

Experimental. The animals used weighed between 190 and 229 g and were divided into 3 groups, one from each group being sacrificed 6, 24, 84 and 168 hr after administration of the plasma. The different groups were given the plasma either intravenously, intraperitoneally or orally, and in the same dose namely 1.5 ml containing 110 mg of protein with 10^5 counts per min per mg. The C^{14}-labeled plasma protein was prepared by feeding the washed and killed *Rhodospirillum rubrum* organisms to 9 donor rats which were sacrificed after 8 hr as in the previous experiments

‡ Our thanks are due to Professor H. A. Barker for growing the organisms used. About a quarter or more of the activity is located in the dicarboxylic acids (Unpublished data of Tabachnick, M., and Canellakis, E.).

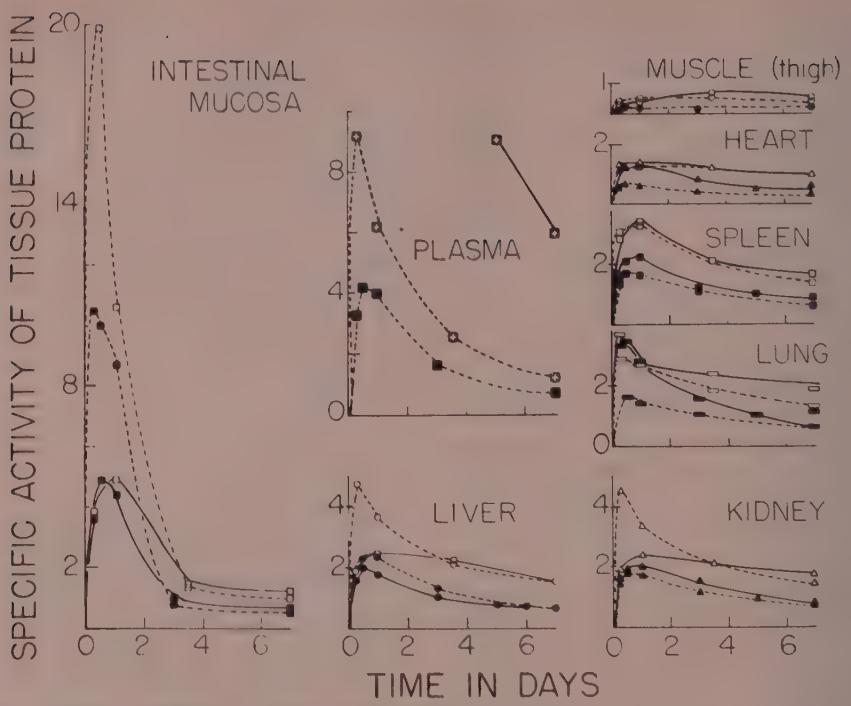

FIG. 3. Specific activities of protein from perfused tissues of Recipients A and B in terms of c.p.m. per mg vs. time in days. Dotted curves after oral administration, solid curves average results following intravenous or intraperitoneal administration. Solid points plasma protein A, open points plasma protein B.

(1). After dialysis of the labeled plasma there was no significant amount of activity in compounds other than protein. Thus 99.8% was trichloroacetic acid insoluble whereas only 0.05% was soluble in the acid; also there was only 0.11% soluble in chloroform. This type of labeled plasma protein will be referred to as 'B', that used in the previous experiments as 'A' *i.e.*, the serine-β-C^{14} labeled plasma protein. All samples of protein, respiratory carbon dioxide, urine and feces collected were subjected to analysis by the procedures previously described(1,2). Specific activities are expressed as counts per min per mg of protein (equals % dose per g protein).

Results and discussion. Loss of label from plasma. The rate of loss of C^{14}-labeled plasma protein from the circulation of animals given the material intravenously is virtually identical with that previously observed in animals injected with labeled plasma protein A. This is shown in Fig. 1 (upper solid curve). Again it appears that more than one process is involved in the loss. The metabolic process which has a half time of 74 hr§ is preceded by a process with a half-time of 11 hr (dotted curve), and by one with a still shorter half-time (not shown). When the same dose of C^{14}-labeled plasma protein is given orally, the amount of C^{14} appearing in the plasma protein of the recipients is rather low, but it will be noticed that it is higher than that previously found with plasma A (Fig. 3). After oral administration the rapid processes

§ From the metabolic half-life of 74 hr we may calculate the rate of synthesis of plasma protein in these 200 g rats as follows: The total plasma protein in the animals is plasma volume x mg protein per ml or 7.5 x 74 = 555 mg. The turnover time = 1.44 x 74 = 107 hr so the amount of protein synthesized is 556 ÷ 107 or 5.2 mg per hr. This represents a minimum rate estimate because no account has been taken of the extra vascular plasma protein. Thus the actual rate is probably 2 to 6 times this value.

found after intravenous administration are not apparent and only the loss of C^{14}-labeled protein from the circulation due to its catabolism is evident. The rate found after 2 days corresponds to that of the slowest process observed following the intravenous injection, and also corresponds with the rate following oral administration of plasma protein A. It is quite understandable that the rates observed for the faster physical processes, should be the same whether labeled plasma A or B is employed, but it does not follow that the rates of the metabolic processes measured should likewise be identical. If plasma protein were to be partially broken down and the peptides or still larger fragments were to be used in resynthesis to any extent then it might be anticipated that different rates would be obtained when different amino acids in the plasma protein were labeled with C^{14}.

It will be noted that there appears to be a more rapid component in the metabolic process, since at the beginning the curve is not linear. The data of Miller and coworkers(3) with dogs and London(5) with human subjects have shown that the half-lives of albumin and globulin are not the same. Consequently a difference is also likely to exist in the rat, so that in a curve for total plasma proteins there will necessarily be a deviation from a straight line in the logarithmic plot even in the part of the curve where catabolism is preponderant. At first slope will be steep due to the contribution of the more rapid process to the rate and later the slope will approach that corresponding to the slower process. It should also be noted that another complication exists because of the unequal specific activities of the albumin and globulin fractions. Similar differences in specific activity have been observed previously(3,4,5) and have been extensively investigated by Yuile and coworkers (6). We do not anticipate that this would greatly affect the results. It is also probable that the rate of loss of albumin and globulin into the intercellular fluid, lymph and cells, proceed at different rates(7). Consequently the more rapid process or processes involved in the loss of plasma protein from the circulation will be complicated in a similar manner to the slower.

When the rate of $C^{14}O_2$ production is examined (Fig. 2) it is seen that it behaves somewhat the same as in the previous experiments(1), which are shown as dotted curves, *i.e.*, the maximum rate of $C^{14}O_2$ production is reached 2 to 3 hr after giving the dose, and then falls off at a rate similar to that observed before. Again there is some delay in attaining the maximum rate after intraperitoneal injection. However, it is obvious that the $C^{14}O_2$ is always less when plasma protein B is used as compared with A *e.g.* in the period 0 to 3 hr the actual figures were for group B (intravenously) 2.1, A 2.7, and for 24 hr B 12.3, whereas A was 15.3%. The animals used were quite comparable so the plasma protein itself was broken down at exactly the same rate in both groups. Even lower rates of $C^{14}O_2$ production have been observed during similar periods in dogs injected with plasma protein labeled by feeding lysine-ϵ-C^{14} to donor animals(5). The difference must be due to differences in the rate of catabolism of the C^{14} label in the free amino acids to carbon dioxide in the several cases.

The difference between plasma proteins A and B is still more obvious when the catabolism of the orally administered material is considered (Fig. 2). With the plasma protein B a very rapid rate of $C^{14}O_2$ production is attained early in the experiments; with A the peak rate is neither so high nor is it attained so rapidly. Consequently there must be some amino acid carbon in the plasma protein B which is metabolized more rapidly than the β-carbon of serine; or possibly the amino acid is released more rapidly from the protein and thus there is a higher concentration of label in the amino acid pool in the one case than in the other. But why the C^{14} from the A protein should be metabolized more rapidly when the protein is given parenterally and more slowly when given orally, as compared with that from the B protein, is not entirely clear. It should, therefore, be emphasized from these results that, although the appearance of $C^{14}O_2$ from C^{14}-labeled plasma protein may serve as an indication of plasma protein breakdown, yet different conclusions may be reached if variously labeled proteins are fed to otherwise identical animals.

Great differences in the amount of C^{14} appearing in the tissue protein are also noted when the results from labeled plasma proteins A and B are compared (Fig. 3). This is particularly true when the proteins are given orally. The labeling of some tissues after protein B may be almost twice as high as after protein A.

It is also noteworthy that following the oral administration of either of the C^{14}-labeled proteins the tissue C^{14} is higher than that attained after parenteral administration. In the latter case part of the activity measured in the tissues must be due to C^{14}-labeled plasma and lymph(2,7). With the protein B lower respiratory $C^{14}O_2$ accompanies the higher tissue C^{14} when the dose is given parenterally. Consequently it is clear from the comparison between these results that little can be deduced concerning the quantities of plasma protein taken up by tissues when the protein is labeled by administering to the donor animals a single labeled amino acid like serine-β-C^{14}. The results obtained at any given time interval will depend on the rate of uptake of the plasma protein by the tissue, its rate of breakdown to free amino acid and on the *rate of catabolism of the particular labeled carbon atom (s) concerned.*

Summary. It is possible to produce generally labeled plasma protein with very high specific activity in rats by feeding them suitable bacteria *(Rhodospirillum rubrum)* cultured in media containing labeled bicarbonate. These bacteria have protein in which all the amino acids present are labeled. The metabolism, in the recipient rats, of the C^{14} in the plasma protein from these donor animals (B) has been investigated and compared with that already described(1,2,7) for plasma protein labeled by feeding donor rats serine-β-C^{14} (A), with the following results: (1) The disappearance of the C^{14}-labeled protein B from the circulation, following its intravenous injection, follows the same curve as that found when protein A was employed. (2) The appearance of $C^{14}O_2$ in the respiratory gases is significantly less in these animals following the parenteral injection, but more following the oral administration than in recipients A. (3) As compared with A, the tissues of recipients B contain more C^{14}-labeled protein no matter which route of administration is employed, and this is particularly true when the C^{14}-labeled protein is given orally.

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Concentration of a Hyperglycemic Factor from Urine of Schizophrenics. (19288)

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The presence of a hyperglycemic factor in the urine of so-called schizophrenic patients has been reported by Meduna and Vaichulis (1). Meduna and McCulloch(2,3) have reclassified these schizophrenics as "oneiro-

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phrenics" and have claimed that a correlation exists between the presence of a hyperglycemic factor in the urine and an abnormal carbohydrate metabolism as demonstrated by a resistance to insulin, a pseudo-diabetic reaction to the Exton-Rose test, and a protracted sugar-tolerance curve during an intravenous glucose-tolerance test.

This study was undertaken to isolate, and possibly characterize, the hyperglycemic factor believed to exist in the urine of patients usually classified as schizophrenics.

Materials and methods. I. *Urine.* Urine from both male and female schizophrenic (without further classification) patients at the Western Psychiatric Institute and Clinic was collected during 4-day intervals, pooled, and preserved in the cold (0 to 3°C) with the addition of thymol. The crude starting-material was precipitated essentially according to Meduna and Vaichulis(1); by acidifying the urine with glacial acetic acid to pH 4.5 and removing the resulting scanty, flocculent sediment by continuous flow through a Sharples supercentrifuge at about 60,000 *g*. Our method for recovering this precipitate consisted in removing the contents of the rotor and homogenizing it in a glass-homogenizer (4) with sufficient water to form a slurry, which was then lyophilized(5). The resulting tan-colored powder (fraction *A*) served as the starting-material for all fractionation studies. During a 13-week period, a collection of about 425 liters yielded 47.6 g of substance *A* (average yield, 0.11 g/l). Individual urine samples varied considerably in *A* content and, in an examination of 4 individual specimens the yield of *A* varied from 0.08 to 0.67 g/l. Harrow *et al.*(6) have described the concentration of a hyperglycemic factor from acidified urine of normals by adsorption on benzoic acid. In order to ascertain whether the same type of material was involved, 25 l of pooled urine from psychotics was treated by their procedure(6), and 5.4 g of substance equivalent to Harrow's Fraction *A* was isolated; (their yield was 4.7 g per 25 l). Although the crude starting-substances obtained by the two procedures were approximately equal in hyperglycemic activity, the Meduna procedure was adhered to by reason of its simplicity and convenience in handling large volumes of urine. However, the benzoic-acid technic has been applied in a preliminary study of individual urine specimens (1 to 3 l), where it appeared to be a superior technic for clinical use.

II. *Bioassay.* The bioassay for induced hyperglycemia consisted in measuring the blood-sugar rise in fasted rats following intraperitoneal injection of the test substance. Male rats (Sprague-Dawley strain; weight 200 to 350 g) were fasted for about 16 hours prior to injection; this period seemed most satisfactory for obtaining low, consistent, fasting levels without a conversion to fat metabolism(7). The usual technic consisted in taking two 0.1-ml blood samples from a lateral tail vein opened by a transverse incision; the first sample, just prior to injection, and the second sample, 100 to 105 minutes after injection. In order partially to overcome the considerable variation in the response of the rats, a semi-quantitative estimate of hyperglycemic potency was obtained by using groups of three rats at several dosage levels. Occasionally, a rat showed little or no response. Most samples were therefore tested several times, and occasional erratic results eliminated from the calculations. It was found that, using a small dose of any of the more potent fractions, the peak of hyperglycemia occurred at 100 to 105 minutes after injection; this is in agreement with the earlier report(1) that the peak occurs at (very approximately) 2 hours after injection.

III. *Blood sugar* was measured by the Horvath and Knehr spectrophotometric modification(8) of the Folin and Malmros method (9) for the determination of blood-glucose in 0.1 ml of blood. We found that this method requires twice as much ferric ion as is recommended. Although the method measures total reducing-substance, its use was justified in that only the magnitude of change, rather than the absolute value, was desired. A semi-quantitative measure of hyperglycemic activity was arrived at by dividing the mean blood-sugar rise, ΔS, by the dosage; thus $\Delta S/mg$, (the mg % rise in blood sugar per mg of substance injected) is an index of potency. The dose-response curve was nearly linear for the

TABLE I. Fractionation and Biological Activity of a Hyperglycemic Factor from Urine of Schizophrenics.

Discard	Outline of processes	Active	Fraction	% from A	Range of doses tested mg/rat*	No. of rats	Potency △S/mg†	Stand. dev.
		Urine						
	Acidify to pH 4.5 and centrifuge							
Supernatant								
		Precipitate	*A*	100	25-85		<1	
	Alcohol and ether extractions							
Supernatant								
		Residue	*B*	90	30-125		1 -1.2	
	Extractions at pH 8 to 10							
Residue								
		Supernatant	*C*	40-45	20-60		1.8-2.7	
	Precipitate at pH 3							
Supernatant								
		Centrifugate	*D*	15-25	6-12	18	3.8	1.5
	Deionize at pH 8							
Resin								
		Effluent	*E*	10-15	5-10	20	4.8	1.7
	Adsorption with Darco G-60							
Charcoal								
		Filtrate	*F*	6-8	4-8	21	7.7	2.4
	Trypsin digestion and dialysis							
Dialysate								
		Non-dialyzable	*G*	4-5	2-4	18	14.4	5.4

* By intraper. inj. See text for studies of intrav. inj.

† Increase in blood glucose above the mean fasting level, divided by mg of material injected, reported as the range for fractions *A* through *C*, and as the means and stand. dev. for *D* through *G*.

lower doses generally used. The mean, pre-injection, blood-sugar concentration in 47 rats was 90.1 mg % with a standard deviation of 5.8. This mean was subtracted from the individual post-injection values to obtain the △S values, because statistical analysis showed the means and deviations to be not significantly different from those obtained by subtracting the individual fasting values from the post-injection rises.

Experimental. A schema of the procedures employed in concentrating the substance, along with the range of biological activities of the various fractions, is presented in Table I. The starting material (Fraction *A*) was not homogenous with respect to solubility in any of the common solvents tested. Of the non-aqueous solvents tried, it was found that alcohol, followed by ether extraction, removed some lipid constituents, including some of the chromogens. The first step, therefore, consisted in two extractions with absolute ethanol (100 ml/g) followed by 2 extractions with ether (50 ml/g); evaporation of the combined alcohol and ether extracts to dryness resulted in a dark-red oil which was emulsified in water with the aid of Tween 80 and, on bioassay, showed no hyperglycemic activity. The al-

cohol- and ether-extracted substance, Fraction *B*, was dispersed in water by thorough mechanical stirring and slight warming to evaporate residual ether. The dispersion was adjusted to pH 8.0 with sufficient 0.1 *N* sodium hydroxide, and centrifuged in Lusteroid tubes at 2400 *g*. The supernatant was removed, the residue redispersed in water, adjusted to pH 9, and again separated by centrifuging. After repeating the process at pH 10, the three extracts were combined (Fraction *C*). For bioassay, the resulting extract (about 50 ml per g of *A*) was neutralized to pH 7 and lyophilized. Otherwise, it was acidified directly to pH 3; this resulted in a fairly heavy, grey, flocculent precipitate which was best separated by freezing the suspension overnight and then allowing it to thaw. The cold, acid supernatant was then readily removed by centrifuging, and was neutralized and lyophilized; this acid-soluble fraction was assayed several times at levels as high as 25 mg per rat, but exhibited no hyperglycemic activity. The acid-insoluble fraction, *D*, was dispersed in water and redissolved by the addition of sufficient 0.1 *N* sodium hydroxide to keep the pH of the solution between 7.5 and 8.0. In preliminary experiments, it was found that uric acid was present in fraction *D*. Since uric acid could be separated from the hyperglycemic factor and since pure uric acid is not active at a 10-mg level, commonly employed for this fraction, a procedure was devised for its removal. The uric acid, and possibly some unidentified ionic contaminants, was removed by batchwise treatment with a mixed cationic-anionic-exchange resin (Amberlite XE-81, Analytical grade) by monobed deionization(10). The resin (1 to 2 g per g of *A*) was added in several portions to a mechanically stirred solution of *D* while the operation was observed with a pH meter. Normally, the pH would rise and then slowly fall to neutrality, at which point the suspension was filtered through a glass-wool pad. This step was repeated when the uric-acid content was high. The effluent (Fraction *E*) was still a dark-amber, somewhat colloidal solution which was biologically active. It was found that this solution could be partially decolorized with adsorbent carbon without loss of activity. Two g of Darco G-60 was added to each 100 ml of a neutral 1% solution of *E*, the suspension heated in a boiling-water bath for 6 to 8 minutes, cooled, and filtered through a Seitz bacteriological filter to remove the carbon and any suspended matter carried through to this step. The filtrate was lyophilized to a pale-grey powder, Fraction *F*.

TABLE II. Elementary Analysis of Various Fractions.

Fraction	*D*, %	*F*, %	*G*, %
Carbon	45.21	44.29	41.41
Hydrogen	6.30	5.89	6.62
Nitrogen (Dumas)	11.37	11.46	8.56
Sulfur	3.85	1.04	.31
Ash	2.93*	6.08	6.81
Cation in ash, calc. as sodium			2.49

* Probably due to fact that this sample was precipitated in an acid solution, whereas *F* and *G* were neutralized fractions.

An elementary analysis of *F* is recorded in Table II. The material gave positive biuret, ninhydrin, xanthoproteic, and Molisch tests. It was precipitated at pH 4.0 to 4.5, but was soluble below and above this region. The active substance was precipitated by sodium sulfate added to 50% saturation. Addition of zinc ions in alkaline solution caused precipitation. The ultraviolet absorption spectrum exhibited but one maximum, at 275 mμ —characteristic of proteins(11). The infrared spectrum exhibited three maxima at 3.0, 6.1, and 6.4-6.5 μ—all of which also occur predominantly in 6 known proteins examined by us and which agree with reported values (12). An ultracentrifugal analysis of substance *F* at 1% concentration in 0.1 *M* phosphate buffer, pH 7.0, gave only a single peak, the sedimentation-velocity constant of which was calculated to be 6.5 Svedberg units at 23°C. All these data are consistent with the tentative conclusion that the hyperglycemic factor is a protein or a material strongly bound to protein. In a number of studies with proteolytic enzymes it was found that *F* could be digested with *crude* trypsin (Armour 1-75), using 4% of *F* at 30-35°C for 2 hours at pH 8, without loss of activity. The digest, in a mechanically-rotated cellophane bag, was dialyzed against distilled water at room temperature for 6 hours. The non-dialyzable

solution was then heated to destroy the trypsin, cooled, filtered, and the filtrate lyophilized. The resulting pale-grey powder, Fraction *G*, which represented about 60% of *F*, was almost twice as active as *F*. The lyophilized dialysate was found inactive at a dosage of 6 mg per rat. *Crystalline* trypsin (Worthington Labs., 1 x cryst.) at a 2% level under similar conditions, produced less dialyzable material and a somewhat smaller increase in potency. The digestion mixture resulting from the action of crude trypsin on purified globulin (Armour) produced no hyperglycemia.

The elementary analysis of *G* is given in Table II. It did not show isoelectric precipitation at pH 4.0 to 4.5, nor was it precipitated by 10% aqueous trichloroacetic acid. The ultraviolet and infrared spectra were practically identical with those of Fraction *F*. The sedimentation-velocity constant was, however, significantly smaller; namely 2.7 Svedberg units at 27°C. On heating *G* in 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide in a boiling-water bath for 10 minutes, the hyperglycemic activity was destroyed. Studies on earlier fractions had indicated that the biological activity of a neutral solution was unaffected by heating in a boiling-water bath for 8 to 10 minutes, and that solutions adjusted to pH 3 or pH 11, prior to heating, lost less than 50% of their activity. The individual steps, as well as the complete fractionation, were repeated a number of times, with minor variations, with essentially the same results.

Results. I. Urine of psychotics. Our studies indicate that there is a substance in the urine of schizophrenics which can be concentrated 15- to 20-fold from a crude, acid-precipitated material and which produces a temporary hyperglycemia in rats when injected intraperitoneally or intravenously. The results, expressed as rise in blood sugar per mg of substance ($\Delta S/mg$), are rather variable for the crude fractions and hence are only expressed as approximate ranges in Table I. The later fractions, *D* through *G*, gave more consistent results. The potencies in a large number of rats, along with the variabilities, expressed as the standard deviations for these fractions, are presented in Table I. With the earlier fractions we noted a considerable degree of toxicity, producing limpness and diarrhea in 1 to 3 hours, and death in 10 to 24 hours in a number of animals. The higher doses of *F* and *G* often produced a transient diarrhea but rarely any other outward signs of toxicity. It was found that intravenous (tail vein) injection gave a potency about 4 times as great as did intraperitoneal injection. The peak of this hyperglycemia came at 70 to 90 minutes, so that, for these bioassays (*i.v.*), the post-injection blood samples were withdrawn after 80 minutes.

II. *Normal urine.* In a study of 21 male and female normals, Meduna and Vaichulis (1) found evidence for the excretion of a small amount of hyperglycemic substance; this gave an average rise in blood sugar showing a maximum at 1 hour after injection and diminishing thereafter. The implication was that the hyperglycemic factor may be a *normal* physiological entity which schizophrenics excrete in abnormally large amounts. Since our study was primarily concerned with the *isolation* of the substance, normal urine was of secondary interest for the present. However, as a control on our procedure, we collected two separate batches of urine from anonymous healthy males at the Mellon Institute. The urine was processed in a manner entirely analogous to that outlined for the urine of psychotics, except that the bioassays were run on fractions equivalent to Fractions *D* and *E*. The results of 3 experiments are given in Table III. As may be noted, very little, if any, hyperglycemia was obtained with the material separated from normal urine, even when tested at somewhat higher dosages than for comparable fractions of psychotics' urine, and this in spite of the fact that the yield of *D* or *E* per liter of normal urine was less than that from the urine of psychotics. Since blood sugar was measured only at 2 hours after injection, the earlier (1 hour), small hyperglycemic effect(1) was not sought for by us.

Discussion. Harrow *et al.*(6) reported on a hyperglycemic substance obtained from normal urine. The blood sugar of fed rabbits (2 kg) was raised 23% by the subcutaneous injection of 2.6 mg of his most active material.

TABLE III. Summary of Data on Urine of Normal Males.

Batch	Vol, liters	Fraction	Yield mg/l	Yield % from A	Bioassay Dosage, mg/rat	Bioassay ΔS/mg
I	44	*A*	230			
		D	14	6.1	11.5, 34	2, .9
		E	2.7	1.2	11, 17	0
II	45	*A*	250			
		E	4	1.6	18, 27	0

Our Fraction *G*, when injected intravenously in fasted rats (250 g) at a level of 0.5 and 1.0 mg/rat produced an increase in blood sugar of 35 and 69 mg %, respectively (which calculates as a ΔS of 17 for a dose of 1 mg/kg). Although the species of test animals and the conditions were different, these figures suggest that the activities of these hyperglycemic substances are probably of the same order of magnitude. However, we obtained at least 20 times as much Fraction *G* per l of urine as did Harrow *et al.* of their most active material from normal urine. Other investigators(13-15) have reported on the presence of a hyperglycemic factor in the pancreas, but, since hyperglycemia is apparently due to several physiological mechanisms, and since the mode of action of our factor is as yet undetermined, speculation on the possible relationship of these various hyperglycemic factors appears unwarranted at this time. Although the specific relationship between the presence of the hyperglycemic factor in the urine and the schizophrenic syndrome requires further investigation, our study lends support to the thesis that an abnormal biochemical pattern may be involved in schizophrenia.

Summary. The presence of a hyperglycemic factor in the urine of some schizophrenics has been confirmed. A scheme for a 15- to 20-fold concentration of the principle is described, along with data on the yield and potency of the various fractions isolated. Physical and chemical studies of the most active fractions indicate the factor to be a protein, or a material bound thereto. By a comparable fractionation of normal male urine, a measurable quantity of the hyperglycemic factor was not found.

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Note on Purification of Human Prothrombin.* (19289)

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Work on methods for the purification of prothrombin has now advanced sufficiently so that products suitable for electrophoresis, ultracentrifugation, and other important studies can be obtained. Large quantities of bovine blood have been utilized for the many experiments performed in the development of these methods(1-3). It is not known whether these methods can be used successfully for the production of human prothrombin.

Procedure. Bovine blood is collected in a special sodium oxalate-oxalic acid anticoagulant(4). Use of the latter makes it possible to keep the ionic strength of the plasma low with the result that prothrombin can be precipitated quantitatively from diluted plasma. Human blood could, of course, also be drawn into such an anticoagulant but we were not in a position to do that for economic reasons. Discarded serologically positive plasma was available in citrate anticoagulant. This is old plasma; however, all of our studies of the past have indicated that prothrombin remains well preserved in such specimens(5-7). The ionic strength of the human plasma was reduced by overnight dialysis against cold distilled water. Pilot experiments showed that a specific resistance of 1,000 ohms was sufficient. Such plasma can be diluted with water and acidified to pH 5.1 and the precipitate will contain practically all of the prothrombin. All other procedures were as previously described (1-3). Arrangements were made to process six liters of plasma at one time, and to complete the work for each preparation in two days. When good results were constantly being obtained with bovine plasma a switch was made to human plasma, and then again back to bovine plasma. In this way, the best possible comparisons of human and bovine plasma could be made.

TABLE I. Yield and Specific Activity of Purified Human Prothrombin Compared with Bovine Prothrombin.

Product No.	Liters plasma used	Total units yield	Specific activity	
			per mg	per mg tyrosine†
510702	6.5	376000	1610	26100
510807	7.5	400000	1780	28000
510808	6.5	264480	1850	28000
510809	7	550000	1800	30300
510813	7	240300	1430	26000
510814*	3	93500	2000	32400
510815	7	614400	—	30000
510816	6.5	366080	—	22130
510820	7	600340	2000	28500
510822	6.5	507840	1860	27130
510823	6.5	438370	1760	25230

* Human prothrombin.

† Method of Folin-Ciocalteau. This is not an accurate analysis for tyrosine but the figures can be compared with previous work from this laboratory. The prothrombin units are the same as previously employed(1-3).

Results. Table I shows the results obtained with 5 lots of bovine plasma and one lot of human plasma, followed again with 5 lots of bovine plasma. It is certainly possible to say that the human prothrombin product possessed at least as much potency as the bovine products, and we believe that the specific activity may be considered to be fully as high. Throughout the purification procedure, it was not possible to detect any gross differences in the way in which the various fractionation steps could be applied to either human or bovine plasma. The lower yield of human material was doubtlessly the result of narrowing the limits of the last fractionation steps. In this way a second fraction of less purity, but with many units of prothrombin, was obtained as a by-product.

Summary. Methods developed for the purification of bovine prothrombin can be applied to the purification of human prothrombin. By these procedures, human prothrombin has been obtained with a specific activity equal to

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the highest ever obtained with bovine material.

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Stability of Purified Hyaluronidase. (19290)

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Highly purified bovine testicular hyaluronidase(1,2) has been found to be quite unstable compared to crude fractions containing less than 1000 turbidity reducing units per mg of nitrogen. McCullagh *et al.*(3) reported inactivation of dried enzyme and aqueous solutions at higher storage temperatures and some stabilization by gelatin and gelatin polypeptides. In this laboratory, however, hyaluronidase suspensions purified to 1,000,000 TRU per mg were rapidly inactivated above 0°C unless specific conditions were imposed. Assays(4) were difficult to evaluate and serious losses were encountered in dialysis, dilution, and lyophilization.

Ranges of pH, ionic strength, temperature, and enzyme concentration that seem to be critical for stability of the highly purified enzyme have been included in this report.

Methods. Hyaluronidase was prepared from fresh bovine testes(1,2) and assayed by the turbidity reducing method(4). Enzyme units were defined by this assay method and enzyme purity has been expressed as TRU per mg N. Enzyme solutions contained merthiolate, 1 to 10,000, and were kept in 0.022 M phosphate-citrate buffer at pH 5.2 containing 0.28% NaCl, ionic strength, 0.10, unless otherwise noted.

Hydrogen Ion Concentration, Table I, was not a critical factor for stability between pH 4.0 and 9.5. However, under less favorable conditions, optimum stability appeared to be between 5 and 7.

TABLE I. Effect of Hydrogen Ion Concentration on Stability of Buffered Hyaluronidase Solutions. Results in TRU/ml.

	Purified hyaluronidase, 60000 TRU per mg N					
pH	3.5	5.5	7	8.5	8.5	9.5
0 day	800	750	950	800	750	920
7	290	855	1020	1030	880	1000
56	200	840	990	930	860	920

Enzyme sol. contained .1 mg protein per ml (of .022 M phosphate-citrate-NaCl buffer solution at −2°C).

Total Protein Concentration, Table II, was found to be the most critical factor, with rapid deterioration apparent at concentrations below 1 mg of total protein per ml. At the same total protein concentrations, purified enzyme was inactivated more rapidly than crude. The high dilutions necessary for assay (4), to about 3 TRU per ml, involved serious errors unless dilutions were made with 0.1% bovine serum albumin solution.

Temperature. Above 0°C the rate of inactivation of hyaluronidase, Table III, was increased with the noteworthy exception that purified enzymes were not more labile at higher temperature if total protein concentrations were high enough.

Buffer Salts and Ionic Strength. This factor played a significant role in the stability of hyaluronidase in aqueous solutions, Table III, and in ethanol suspensions at −2°C, Table IV. Phosphate-citrate buffers were most satisfactory in the presence of .9% NaCl.

TABLE II. Effect of Total Protein Concentration on Stability of Hyaluronidase. Results in TRU/ml.

Protein conc., mg/ml	Crude hyaluronidase, 800 TRU/mg N		Hyaluronidase, 3500 TRU/mg N			Purified hyaluronidase, 48000 TRU/mg N		
	.1	.01	.1	.01	.001	.1	.01	.001
0 day	9.3	1.06	50.2	5.5	.60	750	54.5	6
3	11.4	1.02	50.7	5.7	.30	800	50	1
7	11.3	1.02	50	4.7	.28	810	40.5	.5
56	10.2	.93	35	2.4	.0	643	29	.0

(Stock solutions of the enzyme fractions diluted with .022 M phosphate-citrate-NaCl, ionic strength—.10 at pH 5.2).

TABLE III. Effect of Temperature on Stability of Hyaluronidase Solutions. Results in TRU/ml.

Temp.	.01 mg protein/ml, buffered*				.1 mg total protein/ml, unbuffered			
	Crude enzyme, 800 TRU/mg N		Purified enzyme, 49000 TRU/mg N		Crude enzyme, 800 TRU/mg N		Purified enzyme, 55000 TRU/mg N	
	20	38	20	38	20	38	20	38
0 day	1.24	1.16	46.5	45.2	13.5	12.9	855	855
1	1.17	.89	53.5	52.8	12.4	10.7	800	250
3	1.14	.68	45.2	41	6.2	.0	423	94
28	.64	.0	23.4	.0	.0	.0	200	0

* In .022 M phosphate-citrate-NaCl, pH 5.2, ionic strength .10.

TABLE IV. Effect of Buffer Salts on Stability of Ethanolic Solutions of Purified Hyaluronidase. Results in TRU/ml.

Conc.	Phosphate citrate NaCl, .7 M	NaCl, .15 M	Phosphate citrate, .022 M	HOH	NaAc, .1 M	Borate, .1 M	Phosphate, .1 M
0 day	310	330	270	270	270	290	320
3	340	220	200	250	140	160	140

Purified enzyme, 60000 TRU per ml, suspended in buffer sol., .1 mg protein per ml, at pH 5.2, except borate buffer, pH 7. Solutions cooled to −2°C. Cold ethanol added to .3 mole fraction. (Enzyme conc. at 0 time was 280 to 310 TRU per ml).

TABLE V. Effect of Ethanol on Buffered Solutions of Purified Hyaluronidase at 20°C. Results in TRU/ml.

Conc. ethanol	.1 mole fraction	.15 mole fraction	.2 mole fraction
0 hr	900	760	400
2	800	690	100
24	900	210	10
72	870	155	0

.10 mg protein/ml (of phosphate-citrate-NaCl buffer, ionic strength .10, and pH 5.2).

Purified enzymes could not be dialyzed against distilled water at 0°C without losses as high as 50%; but the same enzymes were satisfactorily dialyzed against 0.9% NaCl, shell frozen, lyophilized, and stored at room temperature for two years without significant loss.

Ethanol concentration. Concentrations up to 0.3 mole fraction, commonly used for purification, rapidly inactivated the enzyme at 20°C, Table V; but caused only minor losses at −2°C, Table IV.

Agitation. Shaking, suction filtration with excessive foaming, and other manipulations that favor a large free surface area usually resulted in significant losses. Five ml of enzyme, 800 TRU per mg N and 0.1 mg protein per ml, shaken for 15 minutes at room temperature by machine were completely inactivated in air as well as in nitrogen. Added albumin, 2 mg per ml, or high concentration of crude enzyme, 12 mg per ml, served to reduce these losses to much smaller value.

Summary. Critical conditions, including pH, total protein concentration, temperature, buffer salts, and ethanol concentrations (for puri-

fication), have been indicated for the stability of purified hyaluronidase.

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Diurnal Variations in Acuity of Sense of Taste for Sodium Chloride.*(19291)

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In normal individuals, diurnal variations exist in acuity of olfaction(1,2) and in acuity of the sense of taste for sucrose(3). The pattern of these variations is remarkably uniform and intimately connected with food intake. Freely selected meals are preceded by a period of increasing and followed by one of decreasing acuity of the senses mentioned. When a meal has been omitted a decrease in acuity cannot be demonstrated. The precibal increase in acuity of olfaction and precibal increase in acuity of the sense of taste for sucrose may represent measurable accompaniments of sensations interpreted as desire for food and the postcibal decrease in acuity of olfaction and postcibal decrease in acuity of the sense of taste for sucrose may represent measurable accompaniments of sensations interpreted as satiety afforded by food. The demonstration of the existence of diurnal variations in acuity of the sense of taste for sucrose made it appear desirable to determine whether or not like variations exist with respect to other taste qualities. The present experiments were undertaken with the purpose of determining whether or not such variations exist with respect to the sense of taste for sodium chloride.

Method and procedure. Gustatory thresholds for sodium chloride were determined by placing a measured volume (0.5 cc) of sodium chloride solution on the tip of the subject's extended tongue. The solutions used had been made up with tap water and contained sodium chloride in concentrations ranging from 0.05% to 0.55% in steps of 0.05%. The lowest concentration of sodium chloride which just sufficed to permit instantaneous recognition of saltiness was interpreted as the measure of threshold for this taste quality for the subject at the time. At each determination the threshold value accepted for the subject was the lowest concentration of sodium chloride which produced the sensation of saltiness 3 times in succession. Before and between successive trials the subjects were requested to rinse their mouths with tap water. In this manner gustatory thresholds for sodium chloride were determined at 10:00 and 11:30 in the morning and at 1:30, 3:00 and 4:30 in the afternoon. Subjects exhibiting coating of the tongue or taking medicine for any reason were excluded temporarily from the experiment. During tests the subjects were kept uninformed as to the concentration of the sodium chloride solutions used. There were 8 individuals, 7 females and 1 male, who served as subjects. They were in apparently good health and ranged in age from 23 to 45 years. They held clerical positions in this institution and worked daily from 9:00 in the morning until 5:00 in the evening. As a rule they had breakfast and dinner at home at customary hours, but ate lunch in the hospital cafeteria which offered a variety of dishes so as to permit reasonably free selection of food. Lunch was served between 12 and 1:00 in the afternoon. On

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test days the subjects were requested to abstain from taking food between meals. Their statements regarding their desire for food as well as their freely selected caloric intake were recorded.

Results. The results presented are those of gustatory threshold determinations performed in all subjects on days (108 test days) on which the subjects observed normal food habits and those of gustatory threshold determinations performed in 5 of the subjects on days (25 test days) on which the subjects omitted their noon meals. Fig. 1 shows the

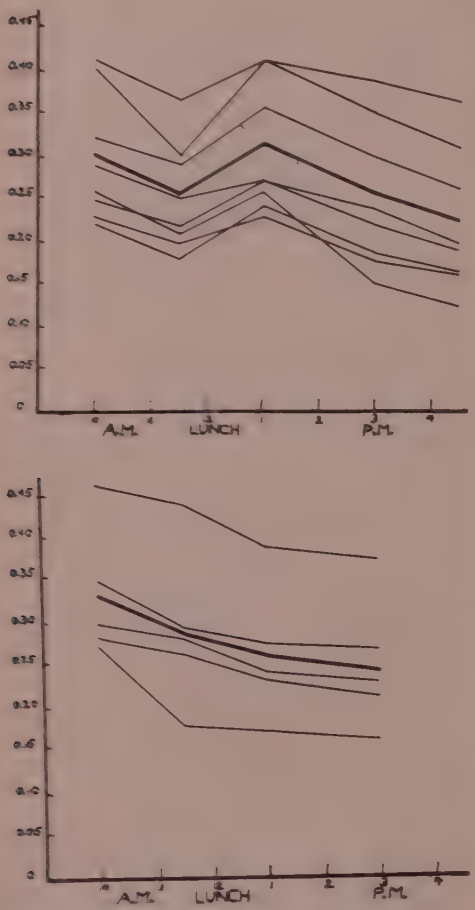

FIG. 1. Upper: Avg gustatory threshold values for NaCl obtained on days when lunch was ingested. Lower: Avg gustatory threshold values for NaCl obtained on days when lunch was omitted. Light lines: for individuals; heavy lines: for the groups. Ordinates: threshold values in % concentration.

averages of gustatory threshold values for sodium chloride obtained at different hours of test days. The illustration demonstrates that the gustatory threshold values decreased during the morning hours, increased following ingestion of lunch and decreased during the later afternoon. In addition, the illustration shows that the increase of gustatory threshold values failed to occur on days on which lunch had been omitted.

Statistical analysis of the results obtained for individual subjects revealed that the average decrease of gustatory threshold values as occurred during the morning hours was significant for all but one of the subjects; that the average increase of gustatory threshold values as occurred following ingestion of lunch was significant for all but one of the subjects; and that the decrease of gustatory threshold values as occurred during the later afternoon on days on which lunch had been ingested, was significant for all subjects. In addition to this analysis of results an inquiry was made as to whether or not the differences between averages of gustatory threshold values obtained for a subject immediately before and 30 minutes after lunch time on days on which lunch was ingested differed significantly from the differences between like averages of gustatory threshold values obtained on days on which lunch was omitted. The results of these calculations are presented in Table I. The values in columns B_1 and B_2 of this table represent the average differences expressed as percentage concentration of solution between threshold values obtained at 11:30 in the morning and 1:30 in the afternoon on days on which lunch was ingested (B_1) and on days on which lunch was omitted (B_2). The values in column P indicate the probability (calculated by means of Student's t value) (4) that the differences between the differences referred to is not significant. Statistical convention justifies the assumption that a difference is significant if the probability that it is not significant is 0.05 or less. The table shows that the difference under discussion was found to be significant for all subjects.

The subject's statements revealed that noon meals were preceded regularly by a period of an increasing desire for food denoted variously

TABLE I. Average Differences Between Gustatory Threshold Values Obtained Before and After Lunch Time (5 Subjects).

Days on which lunch was ingested	Days on which lunch was omitted	Difference B_1 (increase)	Difference B_2 (decrease)	P
19	5	+.04	—.05	<.01
10	5	+.06	—.01	<.01
15	5	+.07	—.02	<.01
13	5	+.02	—.03	<.01
12	5	+.04	—.05	<.01

Expressed as % conc. of NaCl in solution.

by the subjects as a sensation of hunger or one of appetite. Following ingestion of the freely selected noon meals a sensation of satiety developed in all instances while the desire for food vanished. The noon meals selected by the subjects ranged in their caloric value approximately between 300 and 1100 calories. In most instances dessert was eaten by the subjects at the end of the noon meals.

Comment. Changes in threshold values for the sense of gustation indicate changes in gustatory acuity. Thus, decreasing threshold values signify an increase, increasing threshold values a decrease in acuity. Therefore, the observations described reveal that the acuity of the sense of taste for sodium chloride increases during the morning, decreases following ingestion of freely selected noon meals and increases again during the later afternoon. The increase in gustatory acuity during the morning, was noted on 102 (94%), the decrease in acuity following ingestion of noon meals on 86 (79.5%) and the increase in acuity during the later afternoon on 104 (96.3%) of the 108 test days. The analysis of average gustatory threshold values obtained for the individual subjects at different hours of test days shows that the increase in gustatory acuity during the morning was statistically significant in all but one subject, that the decrease in gustatory acuity following ingestion of noon meals was statistically significant in all but one subject and that the increase in gustatory acuity during the later afternoon was statistically significant in all subjects. That the decrease in acuity of the sense of taste for sodium chloride was dependent upon ingestion of food becomes evident from the analysis of results obtained on days on which noon meals had been omitted. This analysis shows for all subjects so tested that the changes in gustatory acuity noted after lunch time on days on which lunch had been ingested differed significantly from those noted at the same time of the day but on days on which lunch had been omitted. It should be noted that this was found to be true also for the one subject for whom the decrease in gustatory acuity following ingestion of lunch was noted to be insignificant.

The evidence presented justifies the conclusion that there exist in normal individuals diurnal variations in acuity of the sense of taste for sodium chloride. Since the freely selected noon meals upon ingestion brought about a conversion of sensations interpreted as a desire for food into sensations interpreted as satiety afforded by food the observations described may be regarded as indicating that the precibal increase in acuity of the sense of taste for sodium chloride may be related to the sensations constituting one's desire for food and that the postcibal decrease in acuity of that sense may be related to the sensations constituting satiety. This interpretation of results is supported by the fact that the decrease in acuity of the sense of taste for sodium chloride failed to occur when conversion of sensations had been prevented by omission of noon meals. The mechanisms by means of which food produces a decrease in acuity of the sense of taste for sodium chloride are being subjected to further investigation.

Summary. Experiments are described demonstrating the existence in normal individuals of diurnal variations in acuity of the sense of taste for sodium chloride. Freely selected meals were found to be preceded by a period of increasing and followed by one of decreasing acuity of that sense. When meals

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Sweat Sodium Levels in Congestive Heart Failure. (19292)

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Evidence has been presented suggesting that overactivity of adrenal cortical electrolyte hormone may be partially responsible for the sodium retention characteristic of congestive heart failure. Biological methods have indicated increases in corticoids(1) and DOCA-like material(2) in the urines of congestive failure patients. Salivary sodium concentrations have been reported to be low in congestive failure(3), also suggesting adrenal hyperactivity. On the other hand Lasche, Perloff, and Durant found low urine 11-oxysteroid and 17 ketosteroid concentrations in the majority of 23 cardiac failure patients(4).

The work of Conn indicates that the activity of the adrenal "electrolyte-influencing" steroids can be correlated with the sweat sodium or chloride concentration(5). Low sweat sodium concentrations (below 10 meq L) are found in hyperadrenal cortical states such as Cushing's syndrome and high concentrations (above 80 meq L) in Addison's disease. Merrill in 1940 stated that several of his congestive failure patients had low sweat sodium concentrations(6); this appears to be the only published reference to the use of the sweat test in evaluating adrenal salt hormone activity in congestive heart failure. In the following study sweat sodium concentrations were investigated by a method applicable to patients with heart failure.

Methods. A plastic jacket with tight elastic neck, arm, and waist bands ensures collection of sweat on the trunk without evaporation. The subject, whose back has been thoroughly cleansed with distilled water, sits in the Fowler position in bed, and is heated by immersing one arm to the elbow in a tub of hot water and by placing several blankets over the body. In from 15 to 90 minutes sufficient sweat will be present on the back to ensure the collection of from 3 to 5 cc when the jacket is opened. A tablespoon is used to transfer the sweat to a test tube. To avoid evaporation it is necessary to wait until enough sweat has accumulated so that the desired amount can be obtained the first time the jacket is opened. Two cubic centimeters are usually sufficient for sodium and potassium determination. The jackets, towels, test tubes, and spoons are prepared by soaking in 3 changes of distilled water, which is sufficient to lower the concentration of sodium in the final wash-water to a negligible level. Blood eosinophil counts using Randolph's stain, were done immediately before the sweat tests. Sodium and potassium were determined with the flame photometer using lithium as the internal standard.

Material. The patients tested were in obvious, severe, congestive failure without evident complicating factors such as infection. All had gross pitting edema. Their ages ranged from 37 to 83 years. The majority of the patients had hypertensive or rheumatic heart disease or cor pulmonale. Some were studied immediately after admission to the hospital for symptoms of congestive failure. Others were studied towards the end of a 24-hour control period designed to standardize as far as

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TABLE I. Results of the Sweat Test in 3 Groups of Congestive Failure Patients.

Patient	Date	Avg temp.†	Na^+ intake, meq.	Urine Na^+, meq.	Fluid intake, cc	Urine vol., cc	Wt change lb	Sweat Na^+, meq.	Sweat K^+, meq.
				Group A, sodium retention					
1. S.	10/4/50	73	31	8.2	2400	430	+1	45.5	6.7
2. C.	9/22	69	22	10	1200	500	0	27	4.9
3. T.C.	11/2	77	22	.65	1700	500	0	69	2.1
4. M.	11/16	58	43	12.6	2500	600	0	49	7.7
5. T.	11/9	61	33	21.5	3000	500	0	55.2	7.6
6. J.	1/6/51	58	33	12.5		600		87	6.4
7. V.	1/6	58	22	15		500		77	5.1
8. D.	1/14	52	22	5.3	1500	700	— .25	37.8	7.8
9. R.	2/6	66	18	6.3	1250	330	+ .5	64	5.1
10. W.	4/3	60	22	3.4	2000	420	+ .75	34	9.2
*	4/14	63	76	112.8	2000	2450	—1	29.5	8.2
11. M.M.	4/12	67	44	23.5	2200	500	+1	51.3	6.4
12. G.	5/6	62	28	17.1	1750	300		45	6.7
*	5/14	61	33		1750	900	0	55	5.4
13. C.R.	6/5	64	55	6.8	5000	800	+3	40	11.6
				Group B, water retention					
14. H.	11/22/50	73	11	46.2	1500	370	+ .25	22	5.6
*	11/30	55	97	122.5	2250	2400	— .5	13.5	5.6
15. D.	1/15/51	55	60		4000		+2.5	31	8.7
16. S.	1/25	73	22	28.8	2500	720	+1.8	22.6	8.2
17. M.S.	4/12	66	33	69.5	2000	600	+ .5	38.3	5.2
18. A.	6/5	64	44	114	3250	1000	+ .8	77	5.2
*	6/11	62	35		2000		0	29	1.2
				Group C, diuresis of both water and sodium					
19. H.	10/20/50	63	55	208	3000	3200	—4	62	11.5
20. F.H.	1/18/51	55	27	62.1	1750	1150	— .25	34	5.6
21. G.	2/5	57	33	51.7	1500	600	— .5	75	6
22. B.	5/13	59	33	130	2500	2000	0	41.7	5.4
*	5/21	61	33		2500	1800	0	22.7	6.1

* Clinically compensated. During the previous 24 hr 4.5 g sodium chloride were given without sodium retention or wt gain.

† Avg of high and low temp. for the day.

possible the fluid and salt intake. During this control period the patients were in bed and received no medications, other than oxygen and opiates when necessary, and, occasionally, maintenance digitalis. Food intake was limited to measured amounts of milk (usually 1500 cc daily) so that the sodium intake could be calculated. The sodium content of milk closely approximated 22 meq per liter. The total fluid intake was recorded and, when possible, the patients were weighed at the beginning and end of the 24-hour periods. The urine volume and sodium content were measured. In 10 patients the test was repeated during a consecutive 24-hour period following the injection of 2 cc of mercuhydrin intramuscularly. Sodium and fluid intakes were essentially unchanged. Sweat tests were performed 8 hours after the injection to coincide with the peak of diuresis. Whenever possible, patients who regained compensation, through rest in bed and the use of digitalis, were restudied during a third 24-hour control period. These patients, on the day before the test, were able to handle a moderate salt load (4.5 g) without sodium retention or weight gain. The *control group* consisted of apparently normal hospitalized patients awaiting hernia repair or biopsy of breast lesions that proved to be non-malignant. They ranged in age from 24 to 60. They were studied during a similar 24-hour control period in which the fluid intake approximated 2500 cc and the food intake consisted of 1500 cc of milk. Three patients receiving ACTH in amounts sufficient to cause a mild Cushing's syndrome, and one patient with Addison's disease, untreated for a week, were tested during 24-hour control periods.

Results. In congestive failure, sweat studies should be most significant if performed when edema fluid is actually being retained. For

TABLE II. Comparison of Sweat Sodium Levels in the Various Groups Tested.

Group	No. of cases	Sweat Na^+, meq./L, mean and S.D.
Controls	18	34.3 ± 17.3
Congestive failure		
Group A	13	52.4 ± 16.5
B	5	38.2 ± 20.2
C	4	53.2 ± 16.2
D	11	33 ± 20.1
After mercuhydrin	10	48 ± 23.3
Compensated	5	29.9 ± 15.4
ACTH treated	3	7 ± .7
Addison's disease	1	117

this reason the congestive failure patients were evaluated by means of the 24-hour control periods. There were 4 groups among the patients studied (Table I). Group A consisted of those patients who were retaining sodium, with oliguria and a low urine sodium concentration, and usually a weight gain during the control period. In Group B were those who were in negative sodium balance with relatively high urine sodium concentrations but who were retaining water as evidenced by oliguria and weight gain during the control period. Group C contained those who, though they entered the hospital in congestive failure, were not actually retaining either sodium or water at the time of the test. The patients who entered the hospital in congestive failure and who were studied immediately without a control period made up Group D (Table II).

In none of the groups of congestive failure patients did the sweat sodium concentration offer evidence of adrenal cortical hyperactivity (Table II). The mean value for the patients with both sodium and water retention (Group A) was, in fact, significantly higher ($P > .02$) than that of the control group, suggesting adrenal hypoactivity. The mean value for Group C was approximately the same although sodium and water were being lost rather than retained. In the few patients who achieved relative "sodium compensation" following rest and treatment, there was a fall in the average value of the sweat sodium concentration. This is further evidence against the presence of adrenal hyperactivity during edema formation.

Mercuhydrin apparently does not affect the sweat sodium. There was no significant difference in the mean sweat sodium concentration of 10 patients before (47.4 meq per liter) and after (48.0 meq per liter) mercuhydrin. The concentrations of sodium below 10 meq per liter in the 3 ACTH-treated patients and the value of 117 meq per liter in the Addisonian patient are similar to the findings of others in such states(5,7).

Blood eosinophil counts and serum sodium levels were determined on all subjects. No correlation was evident between either of these values and the sweat sodium level.

Sweat potassium concentrations are included in Table I. Some desquamation often occurred with sweating even though the back had been thoroughly washed. The solid material is separable from the sweat; however, it is possible that dissolution of some of it may alter the potassium concentration. For this reason the significance of the sweat potassium levels is uncertain.

Discussion. In addition to Conn other investigators have reported the results of analysis of sweat in normals and various disease states(7-10). Sodium and chloride concentrations are roughly parallel so that the concentration of either probably serves as an index of electrolyte hormone activity. Clinically demonstrable disease of the adrenal cortex has been associated with concentrations of sodium below 10 or above 80 meq/L. The normal range of sodium concentration is usually considered to be from 15 to 60 meq/L, but many of the patients in our control group, as well as those of others, have exceeded these limits. This wide range of values in normals serves to emphasize the probability that many factors, both endocrine and non-endocrine, affect the sweat electrolyte composition.

The amount of salt in the diet appears to influence the sodium concentration of sweat (10). In the present study the sodium intake was kept as near 33 meq as possible during the day on which the test was done.

The environmental temperature is a modifying influence on the sweat sodium concentration, presumably through variation in the "tone" of adrenal cortical control. Normals tested in the summer averaged 26 meq/L lower than when tested in the winter in St. Louis,

Missouri(9). The present study was carried out in a relatively mild climate where there was a difference of only 25°F between the average temperatures of the warmest and coldest days on which sweat tests were performed (Table I).

The rate of production of sweat appears to be an important factor affecting its electrolyte concentration. The rate of sweating can be measured on the arm by the method described by Locke *et al.*(10). However, many of our patients were observed to display markedly different rates of sweating on different body surfaces. Some perspired profusely on the forehead and lightly on the back, and others vice versa. If the sodium concentration of sweat is relatively constant on different body areas, as has been assumed, then measurement of the rate of sweat production in any one area might be misleading. Total body sweat volume has been measured by Dill, Hall, and Edwards(11), but the method is not easily applicable to bedridden patients. The back was used in our patients rather than the arm because it was felt that sweat production on the back usually occurred with less heat exposure. The back is poorly adapted to measurement of sweat volume, however. A very gross estimate of the rate of sweating was made by recording the time necessary for the back to become wet. This measurement did not appear to correlate with sweat sodium concentrations.

Many other factors which may affect the volume and composition of sweat, such as parasympathetic and sympathetic tone, blood vessel tone and state of hydration, remain to be evaluated. Therefore it is felt that, at least at the present time, unless the sweat sodium concentration is very high or very low, it probably should not be assumed to be a reliable index of adrenal electrolyte hormone activity.

Summary. (1) Sweat sodium concentrations were determined by a method applicable to sick patients confined to bed. (2) Congestive heart failure patients were studied while retaining edema fluid, before and after mercuhydrin injection, and, in a few instances, when recompensated. There was no evidence that increased adrenal electrolyte hormone activity contributed to the sodium retention of heart failure. The mean sweat sodium concentration of one group of congestive failure patients was significantly higher than that of the controls. (3) Mercuhydrin did not affect sweat sodium concentration. (4) In addition to the adrenal cortex, there may be numerous non-endocrine factors that affect sweat electrolyte composition.

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Active Immunization against Pertussis. Immunization with Live Antigen.* (19293)

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(Introduced by L. E. Holt, Jr.)
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Active immunization against pertussis has made great strides in recent years, due largely to the use of freshly isolated Phase I cultures. There is still some discrepancy in the reports as to its efficacy(1-4), but it is quite clear that neither the degree of protection nor the duration of immunity is comparable to that conferred by an attack of the disease. The inferior immunization power of killed vaccine may be attributed to some alteration of the antigen resulting from the procedure used to kill the organisms. It occurred to us that the use of a live vaccine might be feasible in the case of *H. pertussis*. Since the organism is known not to be invasive, it should not be capable of producing the actual disease when injected at a site other than the respiratory tract. An examination of the literature disclosed two attempts to use a live pertussis vaccine. Nicolle and Connor(5) reported the use of a live vaccine, administered subcutaneously, in the treatment of this disease. They presented no convincing evidence of the efficacy of this procedure; marked local reactions were not encountered. More recently, Lapin(6) carried out intradermal inoculations with live *H. pertussis* in human subjects. He obtained no evidence of spreading infection, but encountered local reactions definitely more severe than those resulting from killed vaccine.

In our studies we undertook (1) to compare the effectiveness of live and killed vaccines in protecting mice, and (2) to explore the feasibility of vaccination with living organisms in man.

Animal experiments. The general plan of these experiments was as follows: White mice, 10-12 g in weight, obtained from Rockland Farms were inoculated intramuscularly with graded doses of living or killed pertussis organisms in equal numbers. After a varying period of time these mice were challenged by intracerebral inoculation(7) of living organisms, and the survival rates studied. It was decided to employ the intramuscular route of immunization, although the intraperitoneal route is most commonly employed when killed vaccine alone is tested. Large doses of live pertussis organism were found to cause toxic deaths when given to mice intraperitoneally. When administered intramuscularly doses up to 1.5 billion organisms in 0.1 ml volume caused local necrotic lesions and death only infrequently. Two sets of experiments were carried out in which the strain of organism used and the killing procedures were varied slightly. In Exp. I a total of 270 mice were used; 180 mice were immunized, 90 mice served as controls. The organism used for immunization was *H. pertussis* strain No. 18323 obtained from Dr. Pearl Kendrick, Mich. Dept. of Health(7). The live vaccine was made as follows: Lyophilized strain No. 18323 was grown on modified Bordet-Gengou Media(8). The 24-hour growth (6th subculture) on this media was removed and suspended in 1% Casamino Acids, P.H. 7.0-7.2. The suspension was centrifuged at low speed to remove clumps of bacteria, and the turbidity adjusted by means of a Klett photoelectric colorimeter to an equivalence of 15 billion org. per cc. Suitable dilutions for inoculation were made from this stock suspension. The dilutions were immediately injected to insure maximum viability. The stock killed vaccine was prepared several weeks earlier in a similar fashion. Merthiolate to a concentration of 0.001% was added, and the suspension stored in an ice chest. It was found to be non-viable after approximately 3 weeks storage at 5°C.

Groups of 30 mice were inoculated with 3 different doses of live and killed organisms and after a period of 21 days were challenged intracerebrally with approximately 100 LD/50 of the homologous strain No. 18323. The

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TABLE I. Experiment I. Protective Effect of Live and Killed Vaccine in Mice.

Immunizing dose, billions	Killed vaccine, survival/total	Live vaccine, survival/total
.06	5/21	5/23
.30	1/21	7/24
1.50	2/25	15/26

All animals were challenged intracerebrally with 50000 organisms strain #18323. Virulence control —LD_{50} (in control mice at age of immunization) = 598 organisms. Age control—LD_{50} (in control mice at age of challenge) = 523 organisms. Non-immunized mice challenged intracerebrally with 50000 organisms, Strain #18323 = 0% survival.

result is shown in Table I.

Among the animals given killed vaccine there was a larger proportion of survivals with the smallest dose than with the larger doses. However, the survival rate in all 3 groups of this vaccine was of such low magnitude that there was no clear dosage-survival rate relationship. The results might therefore be attributed to chance (random) fluctuation. The chi square test was applied to a comparison of the live and killed vaccine and gave the following results:

Dose

0.06—Obviously not significant

0.3 —Not significant at 5% level.

1.5 —Highly significant; $P < 0.001$.

A second series was carried out to confirm this observation and in addition, to determine whether there were any differences in duration of the acquired immune levels. 424 mice were used, 360 being immunized and 64 serving as controls. In this series a freshly isolated strain of *H. pertussis* was employed. The live vaccine was prepared as described above. The preparation of the killed vaccine was varied slightly from the technic previously employed. To a 72-hour Bordet-Gengou culture suspended in saline, Merthiolate was added, and the stock suspension was rendered non-viable at 5°C.

Groups of 15 mice each were injected with specified doses of live and killed organisms and, after periods of 10, 17, 24 and 120 days they were challenged intracerebrally with 50,000 organisms of *H. pertussis* strain No. 18323. The results of this series are shown in Table II.

The data of Table II were subjected to analysis of variance to differentiate between the following sources of variation:

(1) Killed versus live vaccine
(2) Times of challenge
(3) Immunizing dosage levels
(4) Interaction of (1) and (2)
(5) Interaction of (1) and (3)
(6) Interaction of (2) and (3)
(7) Triple interaction, (1), (2) and (3)

The only items that reached the 5% point of significance were No. (1) and (3). For these the variance ratios (Snedecor's F) were: Between killed and live vaccine—F = 9.97; probability between 5% and 1% points. Dosage levels—F = 20.99; probability between 1% and 0.1% points.

If the differences in time of challenge, No. 2, had any effect, it was not great enough to reach the ordinary standard of significance. Both with the live and killed vaccine whatever immunity was attained at 10 days persisted without consistent change at 120 days.

Since all the inter-actions were non-significant, the significant differences between the effects of doses are equally applicable to both types of vaccine and all times of challenge. The significant differences between killed and live vaccine can be taken as equally applicable at all dosage levels and all times of challenge.

The magnitude of the differences which are proved significant in the above analysis can be indicated by pooling the data and estimating percentages of survivals. When the results of the 4 challenges are combined with respect to size of immunizing dose, it is seen that with the smallest dose (0.06B) the substitution of live for dead vaccine increases the survival rate from 11.3% to 21.0%; in the intermediary group (0.3B) the increase is from 29.3% to 59%; and with the largest dose (1.5B) from 53.8% to 65.9%. When all animals of each type of vaccine are pooled, irrespective of dose, the survival rates are: dead vaccine—31.3% and live vaccine—46.7%. The difference is 15.4%.

It was of interest to determine whether the better results with live vaccine were attributable to multiplication of the organism at the site, after inoculation. The following experiment was therefore performed to test this hypothesis. A group of 24 white mice, 15-16 g, obtained from Rockland Farms were inoculated intramuscularly with 200,000 live

TABLE II. Experiment II. Protective Effect of Live and Killed Vaccine in Mice.

Time of challenge, days	Immunizing dose, billions	Killed vaccine, survival/total	Live vaccine, survival/total
10	.06	2/15	4/14
	.30	7/15	9/14
	1.50	6/15	5/14
17	.06	2/15	5/15
	.30	3/15	9/15
	1.50	9/14	7/9
24	.06	0/14	1/14
	.30	1/14	6/13
	1.50	8/14	5/8
120	.06	2/9	2/14
	.30	6/14	9/14
	1.50	5/9	10/10

Time of challenge	Immunizing dose, billions	Killed vaccine— Survival/total	Killed vaccine— % survival	Live vaccine— Survival/total	Live vaccine— % survival
All challenges combined	.06	6/53	11.3	12/57	21
	.30	17/58	29.3	33/56	59
	1.50	28/52	53.8	27/41	65.9
	All doses	51/163	31.3	72/154	46.7

All animals were challenged intracerebrally with 50000 organisms strain #18323.

TABLE III. Total Inoculum—200000 Organisms.

Time killed	Avg No. viable organisms present
30 min	28500
4 hr	5625
7	6975
24	1050
48	82.5
72	0
120	0
144	—

organisms of strain No. 18323 (24-hour culture—suspended in 1% Casamino Acids). At time intervals of 30 minutes to 144 hours, groups of 3 mice were killed, the injected muscle excised, ground, and suspended in 3 cc of 1% Casamino Acids. Aliquot portions were plated on Bordet-Gengou media. After 5 days incubation, the total number of viable organisms was determined. The results are shown in Table III. Viable organisms remained at the site of inoculation over a period of 48 hours but it did not appear that multiplication had taken place.

Human studies. Encouraged by the animal experiments described above we have carried out some preliminary exploratory observations with live vaccine on human subjects, both adults and children. We are now engaged in immunizing a group of children with live vaccine. Results of these studies will be available at a later date. There are obvious practical difficulties in the preparation of a live vaccine for general use. Efforts to overcome these difficulties will depend on the demonstration of efficacy of immunization with live vaccine in the human disease.

Summary. (1) Live pertussis vaccine proved to be more effective than killed vaccine in mouse protection tests. (2) The use of live vaccine in human subjects is under investigation.

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Differentiation of Tobacco Mosaic Virus Strains by Complement Fixation.* (19294)

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Strains of tobacco mosaic virus (TMV) have been differentiated serologically by applying a precipitin test to antiserum previously absorbed with heterologous antigen (1,2) and by determining the amount of virus required to reach equivalence with antiserum(3). Results of the present study show that strains of TMV can also be differentiated by complement fixation in three different ways; first, by determining the amount of virus that reacts optimally with homologous and heterologous antisera; second, by differences in antiserum dilution end-points in homologous and heterologous reactions, and, third, by complement fixation after absorption of antiserum with heterologous antigen.

Materials and methods. Viruses. The type and rosette(4) strains of tobacco mosaic virus and 5 laboratory strains derived by mutation from the type were used. Isolation and symptomatology of the latter will be described elsewhere. Tomato ringspot(5) and Southern bean mosaic (SBM) viruses were included in some tests as useful controls. SBMV was propagated in the Bountiful variety of garden bean. The other viruses were propagated in Turkish type tobacco. *Preparation of antigens.* Virus antigens were obtained by 2 alternating cycles of high (14,000 RPM for 1½ hours) and low (5,000 RPM for ½ hour) speed centrifugation of sap from diseased plants after preliminary mincing and freezing at —20°C. Purified preparations were suspended in fresh physiological saline and stored in small antigen bottles at —20°C. Since the antisera produced by injection with these preparations failed to react with normal tobacco sap, it can be concluded that the preparations contained an insignificant amount of normal tobacco protein. Virus content of the samples was determined by micro-Kjeldahl analysis.† *Preparation of antisera.* Normal serum was taken aseptically before each of 6 rabbits was injected with varying amounts of antigen on the 1st, 10th, 20th, 35th, 52nd, 60th, 78th, and 85th days and bled on the 20th, 35th, 60th, and 100th days. Total amount of virus injected varied from 8.6 mg for the Y24Y1 strain to 134.7 mg for the type strain. Intravenous injections were given exclusively after the 10th day. All sera were inactivated at 56°C for ½ hour. The 10-day antisera had no detectable titer. Hemolysin and complement titrations were carried out as previously described(5), commercial preparations of hemolysin, complement, and sheep red blood cells being used. *Antigen and antiserum titrations.* The optimum antigenic unit is here defined as that quantity of antigen (purified virus) that reacts optimally with a minimal concentration of homologous antibody. The antiserum dilution end-point is defined as the highest dilution of antiserum that completely fixes complement in the presence of an optimum antigenic unit. The optimum antigenic unit and the antiserum dilution end-point were determined by reacting a series of antigen dilutions with 2-fold serial dilutions of antiserum plus 2 full units of complement in all possible combinations for 15 to 18 hours at 4°C, adding sensitized red blood cells and incubating at 37°C for 30 minutes. A typical grid is in Table I. *Cross absorption.* 0.25 ml of heterologous antigen was added to 1 ml of antiserum and incubated at 37°C for ½ hour. The tube was then centrifuged, the supernatant removed, 0.25 ml of antigen again added, and the mixture incubated overnight at 4°C. The tube was again centrifuged to re-

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† The writers are indebted to Mr. David Trkula for carrying out the micro-Kjeldahl analyses.

TABLE I. Data Obtained in Titrating Homologous Antigen and 20-Day Antiserum for the Type Strain of TMV.

Serum dilution	Antigen dilution							Serum control
	1:1000	1:2000	1:3000	1:4000	1:5000*	1:10000	1:20000	
1:10	4	4	4	4	4	4	3	0
20	4	4	4	4	4	4	2	0
40	3	3	4	4	4	4	2	0
80†	1	2	3	4	4	3	1	0
160	+	+	+	1	2	+	0	0
320	0	0	0	0	+	0	0	0
Antigen control	0	0	0	0	0	0	0	

* Optimum antigenic unit. † Antiserum dilution end-point.

move precipitate, and more antigen added. The procedure was continued until no further precipitate was obtained on addition of antigen. The absorbed serum was then inactivated by heating at 56°C for ½ hour and subsequently used in complement fixation tests.

Experimental. The optimum antigenic unit. It is apparent from Table I that the 1:5000 dilution of type strain antigen is the highest dilution that reacted maximally (4 plus) with the greatest range of antiserum dilutions. The amount of virus present in this dilution, based on micro-Kjeldahl determinations, was 2.8 μg and is, by definition, the optimum antigenic unit. When the type and 5 laboratory strains of TMV were reacted with homologous antiserum from rabbits bled on the 20th, 35th, and 60th day following the initial injection, the optimum antigenic unit in each case was independent of the antiserum used, even though the antiserum dilution end-point increased with increasing length of the immunization period, and of the total amount of virus injected into the rabbit. Moreover, in every instance in which a virus strain was reacted with heterologous antiserum, the optimum antigenic unit was the same as that determined from the homologous reaction.

The optimum antigenic units found were: type strain, 2.8 μg; M1 strain, 7.0 μg; 7A strain, 6.4 μg; R1Y1 strain, 5.6 μg; Y21R1 strain, 3.6 μg; and Y24Y1 strain, 3.5 μg. These figures are subject to errors estimated at less than 50%.‡ Thus, there is reason to believe that some of the virus preparations were differentiated from one another by differences in their optimum antigenic units. The question of whether differences observed represent merely differences between virus preparations or real differences between TMV strains has been partly answered by the finding that the weights of virus in the optimum antigenic unit for two additional preparations of the type strain were 2.5 μg and 2.8 μg, respectively.

Antiserum dilution end-points. The 60-day antiserum for the type and 5 laboratory strains of TMV varied in titer from 1:256 to 1:640, the differences being attributable to variation in individual rabbits and not necessarily characteristic of the strains. In some cases, 2-fold differences were found between heterologous and homologous reactions of the same antiserum, but since they were not reproducible, the strains could not be differentiated on this basis. On the other hand, when the rosette strain of TMV was used as antigen to react with antiserum for the type strain, the antiserum dilution end-point was 1:80 as compared with 1:640 for the homologous reaction(4). This 8-fold difference was reproducible and provides a means of differentiating these two strains.

Antiserum for the type strain failed to react with Southern bean mosaic or tomato ringspot viruses. Antiserum for Southern bean mosaic virus reacted with the homologous virus, with an antiserum dilution end-point of 1:20, but failed to react with the type or rosette strains of TMV, or with tomato ringspot virus. The results provide additional evidence for specificity of the reaction.

‡ Duplicate micro-Kjeldahl analyses of the same samples revealed errors of less than 20% while duplicate or triplicate complement fixation tests revealed errors of less than 30% in determining the antigen dilution that contained the optimum antigenic unit.

Cross absorption. Tests were carried out in which samples of antiserum for the type strain were completely absorbed with a laboratory strain to see whether or not antibodies capable of reacting with the homologous antigen would remain. The absorbed serum itself failed to fix complement, showing that no residual antigen-antibody complex could be responsible for the fixation observed when the absorbed serum was titrated with its homologous antigen. Absence of complement-fixing antibodies against the virus strain used for absorption showed that the absorption had been complete in each case. After complete absorption with strains Y21R1, R1Y1, and M1, the type antiserum still fixed complement when mixed with type antigen with antiserum dilution end-points of 1:10, 1:40, and 1:5 respectively, indicating again that these strains differ antigenically from the type. Type antiserum absorbed with strain 7A fixed complement only when antiserum at a dilution of less than 1:5 was mixed with type antigen, indicating that such antigenic differences as exist between the type and 7A strains could not be detected by means of the cross absorption test used.

Discussion. Differentiation of the type and rosette strains of TMV by differences in antiserum dilution end-points in homologous and heterologous reactions and failure to differentiate between mutant strains on the same basis suggest a correlation between genetic and serological relationships. A parallel situation is found in the observation by Knight(6) that strains of TMV can be differentiated on the basis of the kinds and amounts of amino acids they contain, provided the strains are widely separated genetically but not if they are closely related genetically. Whether or not the antiserum dilution end-point method can be used to differentiate between strains only when they differ in amino acid content, or whether the method will always differentiate strains that differ in amino acid content, remains to be determined.

In quantitative precipitin tests reported by Kleczkowski(3), less of the aucuba mosaic strain of TMV than of the type strain was required to reach equivalence with antiserum prepared against the type. This quantitative difference between strains would seem to parallel the difference in optimum antigenic units, and perhaps may be a related phenomenon. In any case, data from the present study indicate that strains differ in the optimum concentration at which they react with antisera, whether the antisera are homologous or heterologous, and that the optimum antigenic unit is a characteristic property of the strain.

If the optimum antigenic unit is independent of the antiserum, then it follows that complement fixation can be adapted for measuring TMV concentration. The reaction is more sensitive than the precipitin reaction previously employed for this purpose and thus should permit studies involving lower virus concentrations. It is suggested that the method might be applied to viruses for which at present there are no adequate biological methods of measuring concentration.

Summary. Tests with purified preparations of 6 strains of tobacco mosaic virus and antisera produced in rabbits with each of these strains show that the complement fixation reaction can be used for differentiating mutant strains of this virus. In some cases, antibody remaining after absorption of specific antiserum with heterologous antigen could be detected by complement fixation in the presence of homologous antigen. In one case, a significantly lower antiserum dilution end-point was attained in the heterologous reaction than in the homologous reaction. Finally, strains were found to differ in optimum antigenic units, in the weight of antigen required to react optimally with specific immune serum. The suggestion is made that complement fixation can be used to measure tobacco mosaic virus concentration.

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Comparison of Biologic Effects of ACTH Protein and ACTH Peptide Given by Continuous Injection.* (19295)

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Reinhardt and Li(1) have reported on discrepancies in the bioassay of ACTH protein and ACTH peptide by the ascorbic acid depletion test(2) and the adrenal weight maintenance test(3). ACTH protein was more potent than ACTH peptide per unit weight in the adrenal weight maintenance test whereas ACTH peptide was more potent than ACTH protein in the ascorbic acid depletion test. These same preparations of ACTH were used in the present studies.

As was shown by Ingle(4), the biologic response to ACTH protein is much greater when it is administered by continuous rather than intermittent injection. It is possible that the apparent differences in the biologic activities of ACTH protein and ACTH peptide are based upon physical differences and hence upon differences in efficiency of utilization under different test procedures. In the present study we have compared some of the metabolic and morphologic effects of ACTH protein and ACTH peptide when given to normal rats by continuous subcutaneous injection. Under these conditions ACTH protein was more effective than ACTH peptide in causing signs of hypercorticalism.

Methods. Male rats of the Sprague-Dawley strain, having an initial weight of approximately 300 g, were used in these experiments. A medium carbohydrate diet (Table I) was fed by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technics and diets were modifications of those described by Reinecke, Ball and Samuels(5). The rat was placed in a metabolism cage which restricted activity so that the animal was unable to reverse its position. A 21-gauge hypodermic needle, having a small barb attached to its shank to prevent withdrawal, was placed subcutaneously. Sterile needles were used for replacement every 48 hours. ACTH protein and ACTH peptide were made up in sterile solutions of 5% degraded gelatin (Plasmoid, Upjohn) at pH of 2.5 and were administered simultaneously in volumes of 2 cc per rat per day by a 6-place continuous injection machine. The gelatin was found to protect ACTH against precipitation and partial inactivation which sometimes occurs when solutions containing it are passed through a polyethylene tubing as was done in these experiments. In separate (unpublished) studies under similar conditions, it was shown that gelatin alone in the amounts used in these experiments is without any significant biologic effect upon the experimental animals. The experiments were carried out in an air-conditioned room at a temperature of 74° to 78°F. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) each day and were preserved with toluene and citric acid (1 g per sample) to insure the acidity of the urine for nitrogen analyses. Urine glucose was determined by the ferricyanide electrode method of Shaffer and Williams(6) and non-protein nitrogen (NPN) was determined by the micro-Kjeldahl pro-

TABLE I. Composition of Fluid Diet.

	g
Cellu flour (Chicago Dietetic Supply House)	60
Osborne-Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Mazola oil + 100 mg vit. K	10
Mazola oil	190
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to total of	2000

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TABLE II. Gross Changes in Rats Given ACTH Protein and ACTH Peptide for 10 Days. 6 rats in each experiment.

Preparation ACTH	Dose, mg/day	No. showing change			Wt 1 adrenal, mg (avg, range)	Wt thymus (avg, range)
			Gross lesions			
		Died	Kidneys	Stomach ulcers		
Protein	.5	0	0	1	39 (31-48)	69 (34-135)
Peptide	.5	0	0	1	35 (32-40)	140 (96-225)
Protein	1	0	5	5	60 (45-74)	36 (24-61)
Peptide	1	0	1	2	38 (34-46)	89 (28-194)
Protein	2	1	4	5	101 (72-124)	28 (22-37)
Peptide	2	0	2	3	70 (50-80)	30 (14-42)

cedure as follows. Proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure; the organic matter was oxidized by sulfuric acid and hydrogen peroxide; the ammonia was distilled off into a standard acid solution and the latter titrated with standard base. ACTH protein was prepared according to the procedure of Li, Evans and Simpson (7) and ACTH peptide was prepared according to the procedure of Li (8). Following an adaptation period of 2 weeks, the rats were given continuous subcutaneous injections of the hormones for 10 days. At the end of this time the animals were anesthetized with cyclopal, exsanguinated and necropsy performed.

Experiments and results. In Exp. 1, 6 rats were given 0.5 mg daily of ACTH protein and six rats were given 0.5 mg daily of ACTH peptide for 10 days. Experiments 2 and 3 were identical except that the daily doses were 1 mg and 2 mg respectively. In each experiment the biologic responses to ACTH protein were greater than were obtained with the peptide.

Survival (Table II). All of the rats survived the experiments except one animal given 2 mg of ACTH protein daily which was found to be dying on the 10th day of injection. *Gross renal damage* (Table II). Some of the rats showed gross pathologic changes in the kidneys which were manifest as a mottled appearance with occasional gray patches and tiny nodules on the surface of the kidney. The nodules represent hypertrophy of the renal tubules. The mottled appearance of the kidneys is believed to represent infection but routine microscopic examination of the tissues was not done in this study. Such changes occurred more frequently among the ACTH protein series. *Stomach ulcers* (Table II). Ulcers occurred more frequently in the rats given ACTH protein than in the rats given peptide. The lesions varied from multiple tiny ulcers which covered the pyloric mucosa to a few deep ulcers which caused hemorrhage into the gut.

Urinary NPN and body-weight (Fig. 1, 2 and 3). All of the rats remained in positive nitrogen balance and gained some weight during the control periods. All of the rats lost weight and developed a negative nitrogen balance during the administration of ACTH protein and ACTH peptide, but the peak of nitrogen loss was not sustained. The extent of average change in weight and urinary NPN was proportional to the dose, and the average change elicited by ACTH protein was greater than that caused by ACTH peptide at each of the 3 dose levels.

Glycosuria (Fig. 1, 2 and 3). None of the rats excreted measurable amounts of reducing substances during the control periods. At a dose of 0.5 mg daily, 4 of 6 rats given ACTH protein and 2 of 6 rats given ACTH peptide excreted glucose on one or more days of the injection period. At a dose of 1.0 mg daily, each of the 6 rats given ACTH protein and 3 of 6 rats given ACTH peptide excreted glucose on one or more days. At a dose of 2.0 mg daily all of the rats on each preparation of ACTH excreted glucose on one or more days. The average level of glycosuria was proportional to the dose and the response to ACTH protein was greater than to ACTH peptide.

Although some correlation between each of the signs of hypercorticalism was evident, there was no perfect correlation between any

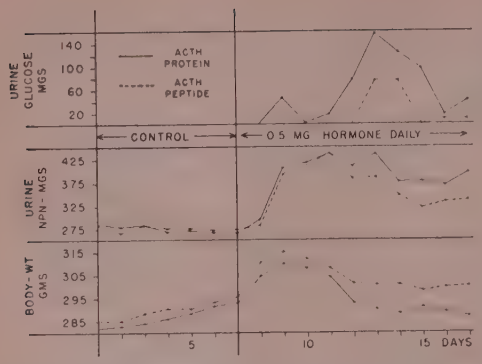

FIG. 1. Comparison of effects of ACTH protein and ACTH peptide given by continuous subcutaneous injection for 10 days. .5 mg/rat/day. Avg 6 rats per group.

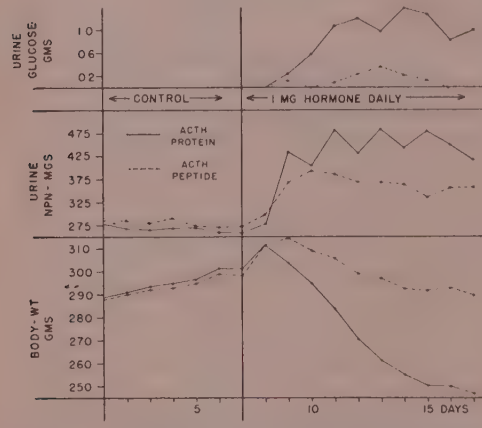

FIG. 2. Comparison of effects of ACTH protein and ACTH peptide given by continuous subcutaneous injection for 10 days. 1 mg/rat/day. Avg 6 rats per group.

two of them, *i.e.*, signs of gross pathology were not limited to the rats with largest adrenals or to animals with the most severe glycosuria, etc.

Discussion. At the time ACTH protein was first isolated(7,9), studies by means of sedimentation, electrophoresis and solubility procedures led investigators to consider it homogeneous. Despite this apparent homogeneity, there is now considerable evidence to indicate that the adrenocorticotropic activity as measured by depletion of adrenal ascorbic acid does not involve the whole protein molecule. It has not yet been possible to characterize fully the active principle or principles in the hydrolysates of the ACTH protein, hence it is not possible to state with certainty that the active component of ACTH protein differs from the active component of ACTH peptide.

The apparent quantitative dissociation in the activities of ACTH protein and ACTH peptide can be interpreted as support for the hypothesis that ACTH represents more than one biologic principle. In the present experiments ACTH protein was found to have about twice the activity of ACTH peptide in causing signs of hypercorticalism. In respect to the adrenal ascorbic acid depletion test(1) the relative potency of these 2 preparations is reversed. At the present time other interpretations of the data cannot be excluded. Differences in the physical properties of ACTH protein and ACTH peptide may cause differences in the efficiency of utilization by the organism and hence in biologic response. The ascorbic acid depletion test is acute whereas the hormone was administered for several days in the present studies. Although the ascorbic acid depletion test has proven useful as a sign of adrenal cortical response to ACTH, its biochemical relationship to the secretory activity of the adrenal cortices is unknown.

In these studies we have controlled one of

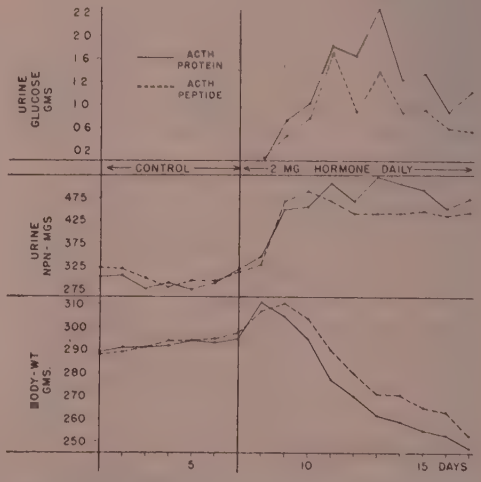

FIG. 3. Comparison of effects of ACTH protein and ACTH peptide given by continuous subcutaneous injection for 10 days. 2 mg/rat/day. Avg 6 rats per group.

the usual variables in bioassays by administering each form of the hormone by continuous injection. Nothing is known of the relative rates of absorption of the two forms of ACTH from the subcutaneous tissues into the blood and of their removal from the blood into the cells of the target organ nor is anything known about the relative proportions of the two forms of ACTH which are inactivated or otherwise wasted before they reach their target organ.

The results of these experiments support the conclusion of Reinhardt and Li(1) that the bioassay of ACTH by the ascorbic acid depletion test does not always correlate with other signs of adrenal cortex response.

Summary. A comparison was made of some of the metabolic and morphologic effects of ACTH protein and ACTH peptide given by continuous subcutaneous injection to normal (300 g) male rats for a period of 10 days. The signs of hypercorticalism were adrenal hyperplasia, thymus atrophy, loss in body-weight, rise in urinary NPN, glycosuria and pathologic changes in the stomach and kidneys. ACTH protein had about twice the biologic activity of ACTH peptide as measured by the above criteria, but was significantly less active than the peptide in causing depletion of adrenal ascorbic acid.

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Is Hypoprothrombinemia Caused by Deficiency of Vitamin K Different from that of Dicumarol?* (19296)

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It is generally accepted that vit. K deficiency causes a true hypoprothrombinemia, and until recently it was believed that Dicumarol likewise reduced only the prothrombin level of blood. One of us(1) obtained findings which were not in accord with this and postulated that the concentration of a factor other than prothrombin might be affected by Dicumarol. This was promptly questioned by Dam(2). Recently, however, he and his associates(3) have postulated that while a decrease of prothrombin occurs in both vit. K deficiency and after Dicumarol, an additional factor is diminished in the first condition, and a second hypothetical agent is decreased after giving Dicumarol. Other workers, likewise, have reached somewhat similar conclusions. MacMillan(4) postulated a decrease of an accelerating factor, while Lein and Lein(5) concluded that Dicumarol causes the formation of an altered prothrombin. Mann and his coworkers(6) recently reported findings which they interpret to mean that Dicumarol decreases a factor, designated "cothromboplastin," much more than prothrombin, while the reverse occurs in vit. K deficiency.

Obviously, there is a need to reinvestigate the problem of whether the hypoprothrombinemia of Dicumarol is essentially different from that of avitaminosis K. Dogs are especially well suited for this study since we(7)

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have shown that in this animal all of the prothrombin is in the active state, thus avoiding the complication of dealing with two types of prothrombin. Vit. K deficiency can readily be produced by a cholecystnephrostomy operation, since the resulting lack of bile in the intestine prevents the absorption of vit. K. That a true hypoprothrombinemia occurs is attested by the prompt restoration of the prothrombin level of the blood when such an animal is given vit. K(8).

Methods. The one-stage prothrombin test was employed. The prothrombin time of normal dog plasma was consistently 6 seconds. Dicumarol was given orally. A dose of 5 mg per kilo of body weight was administered daily until the prothrombin decreased to the desired level. The cholecystnephrostomy operation was done as described by Kapsinow, Engle, and Harvey(9). A dog with this internal biliary fistula remains remarkably well, provided vit. A is given parenterally and the animal is guarded against excessive hypoprothrombinemia. One dog has survived the operation for almost 2 years and has remained in an excellent state of nutrition.

Results. A simple means to determine whether the hypoprothrombinemia of vit. K deficiency is different from that induced by Dicumarol, is to mix the plasmas of both types and compare the prothrombin time of the mixture with that of the two plasmas. When plasma from a Dicumarolized dog having a prothrombin time of 20 seconds is mixed with an equal volume of plasma from a dog deprived of vit. K with a prothrombin time of 31 seconds, the mixed plasma had a prothrombin time of 22 seconds (Table I). The same value was obtained when the first plasma was mixed with a plasma from a Dicumarolized dog with a prothrombin time of 29½ seconds. On mixing the plasma having a prothrombin time of 31 seconds with the plasma having 29½ seconds, the resulting mixture had a prothrombin time of 30 seconds, even though one plasma was from a vit. K deficient dog while the other was from a dog that had received Dicumarol.

These data strongly suggest that Dicumarol, like vit. K deficiency, does not significantly affect any clotting factor other than prothrombin. This is in accordance with our recent studies(11) in which it was shown that Dicumarol acts as an antivitamin K. Since the concentration of the labile factor is not affected by vit. K deficiency, it is to be expected that if Dicumarol acts solely as an antivit. K, it, likewise, will not reduce the level of this clotting agent. This has been shown to be the case experimentally by Quick and Stefanini(12). Recently, Sørbye(3), Owen (13), and their associates have likewise concluded that the labile factor is not decreased by Dicumarol. It is unlikely that other hypothetical factors account for the prolonged prothrombin time resulting from either Dicumarol or lack of vit. K. It seems clear that in the dog the increase in the prothrombin time in both conditions is due to an actual decrease of prothrombin. It is likely that Dicumarol reduces only prothrombin in other higher mammals; but in man, for instance, the problem is complicated, since there is evidence that prothrombin occurs not only in the active form, but also in a precursor state(7,14,15). Preliminary studies indicate that Dicumarol reduces both the free and total prothrombin(16).

TABLE I. Effect of Mixing Plasmas of Dogs Given Dicumarol with Plasma from an Animal Deficient in Vitamin K.

Plasma of dog	Prothrombin time in sec — Observed	Calculated*
A (Dicumarol)	20	
B "	29½	
M (Vit. K deficiency)	31	
A 1 vol. M 1	22	23.5
A 1 B 1	22½	23.7
B 1 M 1	30	30

* On the basis of the standard prothrombin curve(10).

Summary. On mixing equal volumes of plasma obtained from a dog deprived of vit. K and plasma from a dog receiving Dicumarol, the mixture has a prothrombin time that corresponds to the expected average based on the standard prothrombin time curve. It is concluded that in the dog the increased prothrombin time in both avitaminosis

K and after Dicumarol is due to a decrease of prothrombin.

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Effect of Certain Enzyme Inhibitors on Hemolytic and Hemagglutinating Activity of Mumps Virus.* (19297)

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The hemolysin of mumps virus is a labile property with many of the characteristics of an enzyme(1-3). Studies of the effects of certain enzyme inhibitors on the hemolysin were undertaken to obtain further indications of its probable nature and of its similarities to various types of known enzymes. The action of these inhibitors on the hemagglutinating capacity of the virus was tested simultaneously to examine the relationships of the hemagglutinin and the hemolysin or their separate natures.

Materials and methods. A strain of egg-adapted mumps virus was used. 0.1 ml of dilutions of infected egg fluids were injected into the allantoic or amniotic sacs of 6-8-day-old, white, embryonated eggs. The eggs were incubated at 35°C and the infected fluids were harvested 4-6 days later. Allantoic fluids stored at –40°C were dialyzed for 24 hours at 4°C with phosphate buffered saline (pH 7.0-7.2) before use in these experiments. Tests for hemolytic and hemagglutinating activities of the virus were carried out with the methods previously described(2). The enzyme inhibitors were dissolved in phosphate-buffered saline and the pH was adjusted, as necessary, to 7.0-7.2 before addition to the diluents employed in the hemolysin and hemagglutinin titrations.

Results. Urethane(4-7) is a known inhibitor of a variety of enzymes, including several blood esterases. Its effect on the mumps hemolysin is shown in Table I. The inhibition of hemolysis noted with urethane is more marked with the higher concentrations. Using 10% hemolysis as the lowest significant degree of hemolytic activity, 0.1 M urethane decreases the hemolytic titer about 16 times. The 2-fold decrease in the hemagglutinin titer is not significant.

A variety of enzymes, including catalase, are inhibited by hydroxylamine(4-6). The effect of this substance on the hemolytic agent of mumps is shown in a typical experiment in Table I. Hydroxylamine, in a concentration of 0.01 M, was markedly inhibitory for the

* This investigation was aided by a grant from the Division of Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

TABLE I. Effect of Various Concentrations of Enzyme Inhibitors on the Mumps Hemolysin.

Inhibitor	Hemolysis — Dilution of fluid 2	4	8	16	32	64	128	Control	Hemagglutination titer
0	48*	38	33	29	22	16	13	0	1024†
.1 M urethane	21	15	11	9	8	6	4	0	512
.05 M	29	21	19	14	11	7	5	0	512
.01 M	44	25	27	20	18	14	11	0	512
0	40*	39	37	33	34	15	9	0	512†
.01 M hydroxylamine hydrochloride	39	22	12	6	5	1	2	0	512

* % cells hemolyzed determined colorimetrically.
† Reciprocal of highest dilution of virus producing hemagglutination.

TABLE II. Enzyme Inhibitors Which Produced No Significant Effect on the Activity of the Mumps Hemolysin.

Inhibitor	M conc. of inhibitor used
Sodium fluoride	.1; .01; .05; .001
Potassium cyanide	.01; .05; .001
2,4-dinitrophenol	.01
Sodium pyrophosphate	.01
Cystine	.001
Iodoacetic acid	.1; .01; .05; .001

TABLE III. Effect of Reducing Agents on the Mumps Hemagglutinin.

Reducing agent	Diluent	Hemagglutination titer
0	Phosphate buffer	64*
.01 M cysteine	" "	4096
.05 M glutathione	" "	4096
0	Saline	128
.1 M cysteine	"	4096
.05 M glutathione	"	4096

* Reciprocal of highest dilution of virus producing hemagglutination.

hemolysin in all dilutions greater than 1:2. It reduces the significant hemolysin titer more than 8-fold. No effect was noted on the hemagglutinin.

Another group of substances which affect a variety of enzymes was tested for their effects on either the hemolysin or hemagglutinin of mumps virus as listed in Table II. They were without effect on either property of the virus under the conditions of the tests and in the concentrations noted.

Since the virus rapidly loses its hemolytic activity with ordinary methods of handling, it was thought that aeration might play a role in the process, but oxygenation of virus-containing fluids with pure oxygen for 75 minutes at room temperature was without effect on the hemolysin. To study the effects of reduction of the O/R potential on the viral hemolysin various reducing agents, such as cysteine and glutathione, were added to the diluting fluids at the time of the titration for hemolytic and hemagglutinating activity. In these experiments, the tubes were tightly stoppered to insure maintenance of reducing conditions. Evidence was obtained that the hemolytic component was somewhat inhibited by 0.05 M glutathione but 0.01 M, 0.05 M, and 0.001 M cysteine generally produced some increase in hemolytic action. However, both reducing agents produced a marked enhancement of hemagglutination as noted in Table III. Neither cysteine nor glutathione had any effect on the erythrocytes *per se* with the exception of causing some reduction of the hemoglobin. Other reducing agents, such as 0.1 M hydroquinone and 0.06 M sodium sulfite had a similar enhancing effect on the hemagglutinating activity of the virus.

Discussion. In certain of its properties, the hemolysin of mumps virus resembles the hemolytic agent, lecithinase A(4-7). Both the mumps hemolysin and lecithinase A are inhibited by urethane(4-6) and calcium and magnesium ions(5,8). At an optimal pH range 7.0-7.2 and a temperature of 37°C, the action of either of these agents results in the lysis of red blood cells. Neither agent is affected by potassium cyanide or sodium fluoride. However, lecithinase A is heat stable whereas the mumps hemolysin is relatively heat labile(2).

Inhibition of mumps virus hemolytic activity without a corresponding decrease in the hemagglutinating potency by urethane and hydroxylamine hydrochloride gives further evidence for the separate identity of the

hemolysin and the hemagglutinin. The striking enhancement of the hemagglutinating capacity of mumps virus by reducing agents which may actually suppress hemolytic activity also suggests that they are distinct entities. Lowering the O/R potential apparently provides optimal conditions for the agglutination of erythrocytes by the virus.

Summary. Mumps virus hemolysin is inhibited by hydroxylamine hydrochloride and urethane without affecting the hemagglutinin. Cysteine increases both the hemagglutinating and hemolytic activity of mumps virus while glutathione, which has a marked, potentiating effect on the hemagglutinin, inhibits the hemolytic activity. Other reducing agents, such as hydroquinone and sodium sulfite, increase the hemagglutinating capacity of the virus. The different effects of these various substances on the hemolysin and the hemagglutinin suggest that they are distinct entities. The possibility that the hemolysin of mumps virus is a lecithinase is discussed.

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Saphenous Circulation Time Test with a Radioactive Tracer. (19298)

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In January, 1947, routine post-operative stimulation of calf muscles was instituted at Crile V.A. Hospital, Cleveland, with the object of reducing the incidence of thrombosis and embolism(1). In order to find a possible explanation of the apparent good results obtained with this treatment, tests were done to ascertain the effect of sinusoidal stimulation of calf muscles upon circulation time. With the use of sodium cyanide as a test medium it was shown that sinusoidal stimulation of calf muscles reduces circulation time(2). However, the methods of study of circulation time have in the past necessitated the entry of the test medium into the systemic circulation. The test being described in this report, through the use of radioactive iodine and a scintillation counter, permits the study of circulation time in a vein or even part of a vein. The present report deals with circulation time studies in the great saphenous vein.

Test solution. Radioactive iodine (I-131) was chosen as being particularly suitable for this procedure. It is readily available, being supplied by the Oak Ridge National Laboratories and flown to the hospital at frequent intervals for use in the diagnosis of thyroid disorders. Its half-life of 8 days is long enough for an adequate stockpile to be maintained. The dose presently employed, 20 microcuries, is strong enough to permit recording by a scintillation counter, and small enough to allow as many as 5 tests at one session without exceeding the maximum total dose considered desirable in such a diag-

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nostic procedure. With a more sensitive scintillation counter which has recently been tested the dose can be reduced to as low as 5 microcuries, and thus permit as many as 20 tests on the same patient at one session. *The scintillation counter.* The measurement of saphenous vein circulation is accomplished by detection of the gamma radiation of I-131. An insignificant amount of the beta radiation from this isotope is capable of penetrating the tissues which overlie the femoral vein. With a scintillation counter, gamma activity is readily countable when relatively small individual doses are injected. The detecting mechanism constructed according to the design of MacIntyre(3) is an anthracene crystal positioned between the photo cathodes of two 1P21 photomultiplier tubes. Upon absorption of gamma radiation, the crystal produces a characteristic fluorescence which is detected by the photomultiplier tubes. The pulse resulting is amplified and fed into a scaler where counting of the pulses is accomplished. The counter itself is shielded with the equivalent of 1″ of lead on all sides, except directly over the crystal, where the total shielding is that of 3/32″ of brass, the lighttight container of the crystal. The crystal, then, is effectively exposed to the counting site, through a lead collimation 1″ in length and 2″ in diameter, giving a total field of approximately the circular area. The counter is placed in contact with the skin over the femoral vein at the groin and background counting is initiated. Following the establishment of stable background, injection is made at time zero. Counting is continued for approximately 2 minutes during which time the count is first seen to rise sharply with the arrival of the injected isotope under the counter, then decrease as the main body of the isotope passes the counter, and then, in some cases, to rise again if an appreciable amount of the unmixed isotope flows under the counter in the femoral artery. This return is not always discernible, especially in cases of increased circulation time, because of mixing of the isotope in the circulatory system. In order that continuous records of the background, injection point, and rise times may be made, scaler pulses from the counter are fed to a count rate

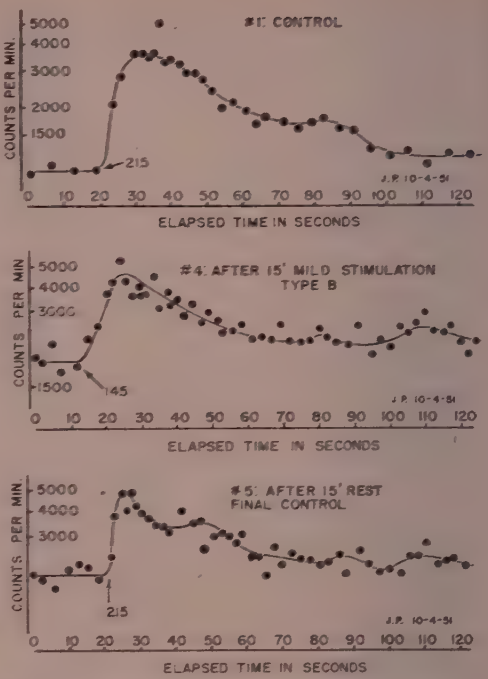

FIG. 1. Plotted curves of saphenous circulation time, before and after 15 min sinusoidal stimulation of calf muscles.

computer (Berkeley Model 1600) which is equipped with output to a continuous strip chart recorder of the Esterline-Angus 0-1 ma type. Timing is accomplished by use of the chart itself, and is sufficiently accurate for the experiment with a chart speed of 12 inches per minute. Once the chart record of a given administration is completed, the data are taken from the chart and plotted as total count vs. time, thereby giving the curves shown (Fig. 1) from which a final evaluation of circulation time is determined.

The shapes of individual curves show some variation due to more or less mixing of the isotope, *i.e.,* in cases of long circulation time when mixing is necessarily more complete, the initial rise in counting rate is of lesser slope, the peak of the curve is lower, and the effective length of time for passage of the isotope is increased, in comparison to that of shorter circulation times, when the peak is of greater slope, greater maximum and of shorter duration.

The bed. A well padded physical therapy

TABLE I. Saphenous Circulation Time.

Patient	Date	Vol. inj. (cc)	Test time in sec 1st	2nd	3rd	4th	5th	Room temp., °C
Group I: Continuous flow of i.v. saline after inj., 60-120 drops/min								
N.	8/9/51	1.00	14	12				28
T.	24	.30	29	28				22
C.	9/12	.22	25	17	15			27
M.	13	.24	16	17	17			28
B.	20	.14	34	31				29.5
W.	28	.16	85	82				25.5
P.	10/ 4	.27	21	21				31.5
G.	5	.29	10	9	7	7	8	32.5
E.	9	.23	13	18	22	21		26
E.C.	9/11	.20	20					28
	27	.26	23					27
Group II: I.V. saline stopped after inj. until a value had been recorded								
J.B.	10/15/51	.40	67.5	70				28
S.	15	.40	25	15	15			28
L.	17	.24	46	45	42			27
F.P.	18	.27	107	98	107	112	107	27
G.	22	.38	20	20	20	20		25

Five successive tests were performed at a single session at intervals of 5 to 20 min. Patients W., J.B., L., and F.P. had known disorders of venous or arterial circulation which would be expected to interfere with blood flow. Remaining patients were normal. First readings on C. and S. were made prior to usual 15 min period allowed for stabilization of flow after insertion of needle.

treatment table has been found suitable for the purpose of the studies. It is comfortable and allows freedom of motion on all sides. The room should preferably be of constant temperature and humidity. Lacking such, we have been using a partitioned off corner of a large room in the Radioisotope department. The temperature of this room has varied from day to day, but for the duration of each experiment, which has taken as long as 2½ hours, the temperature has not varied more than ½°C. *Needle and saline solution.* A 20-gauge needle is used. It is kept open by a continuous flow of normal saline solution at the rate of 60 drops per minute. A 3-way stopcock allows the introduction of saline or test solution as required.

Preliminary preparation of patient. In order to avoid absorption by the thyroid of appreciable quantities of radioactive iodine, Lugols solution is administered by mouth prior to the test. A dose of 10 drops is given at 6 and 10 p.m. the day before and again at 10 a.m. the day of the test. The patient is brought to the department at 12:30 p.m. The mean value of thyroid uptake after 24 hours for 21 patients tested was 2% of the total dose, with a range from 0% to 16% and with only 3 cases being in excess of 10%. The maximum value of 16% uptake occurred in only one case. On comparison of this maximum with that of 35% for normal uptake with 50 microcurie tracer doses, it is evident that the dose delivered to the thyroid in these experiments is equal to or much less than the dose delivered in routine diagnostic studies of thyroid function. Similarly, applying the method and values of Marinelli (4) and assuming a 70 kg man, with a 24-hour urinary excretion of I-131 of 65% of the total dose, it can be shown that the integrated total body radiation delivered in these experiments is only in the order of 10% of the daily tolerance of 0.05 e.r. (equivalent roentgens).

Procedure of test. A tourniquet is applied to the calf. The needle, held in a small empty sterile syringe, is inserted into the dorsal vein of the foot, which is the beginning of the great saphenous vein. The appearance of blood in the syringe indicates entry into the vein. The tourniquet is released, the syringe withdrawn, and the stopcock leading to the intravenous saline is connected with the needle. By the use of an adjustable clamp, the rate of saline flow is regulated to 60 drops per minute. At least 15 minutes are allowed

for stabilization before the first test dose is given. A point is selected under Poupart's ligament ¼" medial to the femoral artery, the pulsations of which can be felt. This point corresponds to the femoral vein just proximal to the junction of its great saphenous vein tributary. The point is marked with ink. Over this point is placed the sensitive chamber of the scintillation counter in such a manner that it is as close to the skin as possible without pressing upon it. The strip chart record is set in motion. When the background reading has been stabilized the flow of saline is discontinued and radioactive iodine is injected during an interval of less than 1 second. The time of injection is marked by hand on the moving paper of the recorder with an error in timing estimated to be less than ½ second. Since the time of flow is never less than 7 seconds a mechanical signal device is not essential. In one series of patients (Table I, Group I) the saline was allowed to resume its flow immediately after the test injection. In another group (Table I, Group II) no saline was allowed to flow until after the reading was recorded. The latter procedure would appear to be preferable in that a possible accelerating effect due to the flow of saline is avoided.

The circulation time having been obtained and permanently recorded, various modalities can then be applied to the calf or any other part of the body, and their possible effects upon this circulation time can be ascertained. Table I shows the control circulation times as obtained in patients before the administration of any physical modalities intended to promote venous flow.

Discussion. The saphenous circulation time appears to be reasonably constant for the same individual tested at intervals of 5 to 20 min. One patient (E.C.) showed no change in time when retested after 16 days. Preliminary tests already made, as indicated by one example (Fig. 1), show that saphenous circulation time is affected by various modalities or even by the same modality differently administered. It is hoped in the course of time to study a sufficient number of patients to permit an evaluation of such modalities upon the saphenous circulation time as measured by the present technic.

Conclusion. A saphenous circulation time test using radioiodine which is detected by a scintillation counter has been described, and its possible uses have been indicated.

We wish to thank Dr. R. A. Shipley, Director of the Radioisotope Unit of Crile V. A. Hospital for his constant cooperation and guidance in the development of this test.

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Acute Arterial Plethora. (19299)

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Byrom and Dodson(1) reported the production of acute necrotizing renal arteritis in 10 of 23 young rats within 3 days after an acute transient episode of hypertension, which had been induced by a series of rapidly administered intra-arterial injections of Ringer's solution. The vascular lesion was described as an acute necrotizing arteritis and was usually limited to one or 2 vessels in each kidney section. The authors concluded from their experiments that a sudden brief rise in intra-arterial tension can cause fibrinoid arterial necrosis in a normal animal and that the smaller arteries of the kidney are selectively

vulnerable to injury in this manner. In view of these findings and the possibility that such a mechanism may operate in the production of the necrotizing vascular lesions seen in malignant hypertension in man, it was felt that these experiments should be repeated.

This report deals with the acute phase of the experiment. The long-term results of a single episode of increased intra-arterial tension will be reported later.

Experimental. Two groups of rats, one comprised of 6 animals 5 months of age, and one of 6 animals 2 years of age, were subjected to acute hypertensive episodes in a manner similar to that employd by Byrom and Dodson. They were of the Sprague-Dawley strain. Intraperitoneal pentobarbital was used instead of ether for anesthesia. Another minor variation was that half of the animals were injected with normal saline and half with Ringer's solution, whereas Byrom and Dodson used Ringer's solution exclusively. The vagus nerve, and, when possible, the sympathetic nerves were dissected off the carotid artery. A cannula, which was made from a 22-gauge needle, was directed proximally into the carotid artery. The contents of a 2 cc syringe were emptied with greatest possible force into the artery. Each syringeful was injected in less than 2 seconds. The solutions were injected at a temperature of 37°C. The number of injections per animal varied between 4 and 14, and the intervals between injections varied between 5 and 15 seconds. The cannula was then removed, the artery ligated, and the incision repaired.

Several animals showed a drooping of the eyelid on the operated side. This was thought to be due to interference with the sympathetic chain. There was obvious respiratory distress with wheezing and dyspnea during and following the injections. This tended to be less severe after 30 minutes. A marked diuresis occurred within 3 minutes after the injections of saline or Ringer's solution. After 3 days the animals were killed, one-half by intraperitoneal injection of pentobarbital, and one-half by ether inhalation. Gross examination of organs was made and the entire animal placed in formalin for fixation. Microscopic examination was done independently by each of us as a method of checking observations. Most organs were examined and multiple sections of both kidneys of each rat were studied. The sections were stained with hematoxylin and eosin. Sections of kidneys were also stained for fat.

Effect of intra-arterial injection of fluid on blood pressure. A preliminary exploratory experiment was undertaken to investigate the nature and magnitude of the rise in arterial pressure that occurs in the rat incident to rapidly repeated 2 cc injections of fluid into the arterial system. Normal adult rats were anesthetized by intraperitoneal injections of sodium pentobarbital, after which a blood pressure cuff was placed on one of the hind limbs of the animal. Systolic pressures were recorded by means of a photoelectric tensometer(2-4). Injections of 2 cc of fluid into the carotid artery were then made and blood pressures were recorded during and immediately after the injections. Six animals were tested in this manner. In each instance the animal was normotensive prior to injection. In no animal was there an increase in systolic pressure, though as much as 18 cc of fluid were injected in repeated 2 cc amounts. The average time interval between injections was 10 seconds. Although this is an indirect measurement of blood pressure it has the advantage over direct aortic measurements as used by Byrom and Dodson(1) in that there is no disturbance of the circulation except for the one carotid artery used for cannulation and injection of fluids. It permits recording of pressures in branches of the arterial tree which more closely parallel the pressures transmitted to visceral organs. From these results it follows that no marked increase in pressure is transmitted far beyond the aorta and that in the normal rat the compensatory changes to increased fluid volume of the vascular bed are immediate.

Pathological changes encountered in rats three days after experimental induction of acute arterial plethora. The total amount of fluid that was injected into each animal of the younger age group (5 months) was: 16, 18, 18, 18, 18, and 20 cc. A careful search of multiple sections disclosed no vascular abnormalities in the kidneys of any of the 5-

months-old animals. Moderate to marked passive hyperemia was observed in the liver, spleen, kidneys and lungs. In one animal there was a recent, small intra-alveolar pulmonary hemorrhage. The total amount of fluid that was received by each animal of the older age group (2 years) was: 10, 16, 18, 20, 24, and 28 cc. Acute necrotizing renal arterial changes of the kind described by Byrom and Dodson were not observed in any of these animals. However, all of these animals were found to have focal or diffuse chronic pyelonephritis with renal parenchymatous atrophy, interstitial exudation and fibrosis and chronic degenerative and proliferative changes in both arteries and arterioles in the regions of inflammatory change. Examination of the other organs of these animals failed to disclose evidence of acute vascular injury. The only change observed was hyperemia of liver, spleen, kidneys and lungs.

Pathological changes encountered in control series. Eighteen rats served as a control series. Six of these were approximately 5 months of age and 12 were approximately 2 years of age. These were presumably normal animals that were sacrificed for the purpose of determining whether or not the pathological changes observed in the experimental group were significantly different from those which occurred spontaneously in animals of the same strain and of comparable age which had been maintained on the same diet and in the same environment. No renal or arterial abnormalities were observed in the 6 young (5 months) animals. In 7 of the 2-year-old control animals pyelonephritis and associated vascular lesions similar to those observed in the older experimental group were seen. The incidence of active degenerative and vascular changes in the control animals was comparable to those seen in the experimental group.

Summary. (1) These experiments were carried out in an effort to confirm a previous report of the production of an acute arterial and arteriolar necrosis in the rat by sudden brief increases in intra-arterial pressure. Twelve rats were subjected to such an experiment. These animals were given rapid injections of saline or Ringer's solution into the common carotid artery. Increases in peripheral arterial pressure during and immediately following injections of fluid were not detected by indirect measurement of blood pressure. (2) In no instance was there evidence of vascular abnormalities other than those which could be explained on a basis of pre-existing pyelonephritis. The existence of pyelonephritis in both experimental and control groups depended on the age of the animals and was seen exclusively in the older age groups. There was no correlation between the vascular lesions and the total volume of fluid injected in the experimental group. (3) Neither acute necrosis nor inflammation of the arteries or arterioles was produced in the rat by a sudden brief episode of hypervolemia. The brief rise in intra-arterial pressure that occurs during the rapid injection of fluid does not produce detectable changes in peripheral arterial pressure. These observations are not in accord with the report of Byrom and Dodson.

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Antifungal Properties of the Polymyxins. (19300))

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Previous investigations with the various polymyxins have established the marked effectiveness of these antibiotics against Gram-negative bacteria(1-3). In view of the antifungal properties possessed by certain aliphatic fatty acids, the isolation of a fatty acid component from the polymyxins(4) suggested a study of these antibiotics for similar properties. Identification of the acid as an isomer (6-methyl-octan-1-oic acid) of pelargonic acid (5) added further interest in light of the work reported by Rothman and co-workers(6,7), who found pelargonic acid to be quite prominent in a group of highly active normal aliphatic monobasic acids isolated from hair fat of adults.

In the present study, the relative antifungal activities have been determined for polymyxins A, B, D and E. Several known antifungal agents were included for purpose of comparison. While the investigation was in progress, Serri(8) reported on the activity of areosporin (polymyxin B) against a number of dermatomycetes. The antibiotic showed greatest activity against *Achorion schoenleini,* was less active but equally so against *Trichophyton gypseum asteroides, Microsporum audouini, Microsporum felineum* and *Sporotrichum gougeroti,* and least active against *Trichophyton rubrum, Trichophyton crateriforme* and *Achorion quinqueanum.*

Experimental. Preliminary determinations of the antifungal properties of the polymyxins* were carried out by means of the agar cup-plate method of Burlingame and Reddish (9). A serial dilution technic was used to ascertain fungicidal activity. Known antifungal agents included in the study were: benzoic acid, salicylic acid, undecylenic acid, G-4† and Asterol.‡ *Fungistatic procedure.* Sabouraud's maltose agar was used in the agar cup-plate method, each agent being tested in plain medium and in the medium containing 10% normal horse serum. The diameter of the cup was 1.5 cm. Inocula consisted of 5-day-old cultures of the respective test organisms, *i.e., Candida albicans, Trichophyton mentagrophytes* and *Debaryomyces neoformans.* Solutions of each agent were made up at a concentration of 0.1% in 95% ethyl alcohol, from which 0.1 cc was pipetted into the cup. Control tests on the alcohol solvent were included in each run. Plates were covered with unglazed clay tops and incubated for 5 days at 28°C. The extent of growth inhibition was measured in millimeters from the edge of the cup to the periphery of the cleared zone. *Fungicidal procedure.* The medium consisted of 1% neopeptone and 4% maltose, adjusted to pH 5.6. As in the fungistatic procedure, determinations were made in both plain medium and in broth containing 10% normal horse serum. Stock solutions of the respective test materials were prepared in 95% ethyl alcohol at a concentration of 0.8%. Subsequent dilutions were made aseptically in tubes of broth so that each drug was assayed at a maximum of 25 mg %, with lower concentrations decreasing by halves. Each tube contained a final volume of 10 cc of medium plus drug at the various dilutions. Although controls on the alcohol solvent were included in each series of tests, it was found in repeated determinations that the concentration of ethyl alcohol in broth necessary to inhibit growth of the various test organisms safely exceeded that present in the initial tube of the dilution series.

The following organisms were used in the fungicidal tests: *Candida albicans, Trichophy-*

* Polymyxin B as the sulfate was kindly supplied by Chas. Pfizer and Co. and assayed at 7625 U/mg. The remaining polymyxins were prepared in our laboratories as the hydrochlorides, by Dr. H. A. Nash with the following potencies: polymyxin A, 9425 U/mg; polymyxin D, 1526 U/mg; polymyxin E, 9500 U/mg.

† G-4, trademark of Sindar Corp. for bis (2-hydroxy-5-chlorophenyl) methane.

‡ Asterol, trademark of Hoffmann-LaRoche, Inc. for 2-dimethylamino-6-(β-diethylaminoethoxy) -benzothiazole dihydrochloride.

TABLE I. Fungistatic Activity of Polymyxins and Various Known Agents.

Agent	Zone of inhibition in mm — *C. albicans*		*T. mentagrophytes*		*D. neoformans*	
	S*	NS†	S	NS	S	NS
Polymyxin A	2	2.5	.5	1	.5	2
B	3	3	2.5	3	4.5	4
D	2	2.5	.5	1.5	1.5	1
E	2.5	2.5	2.5	3	4.5	4
Benzoic acid	2	1.5	1	2	.5	2
Salicylic acid	1.5	2	0	1.5	2	2
Undecylenic acid	2.5	3.5	4	10	2	4.5
G-4	4	7	4.5	15	3	10.5

* Medium plus serum. † Plain medium.

ton mentagrophytes, Trichophyton purpureum, Microsporum audouini and *Microsporum lanosum.* Cultures grown on Sabouraud's maltose agar at 28°C for 14 days served for the inocula. These were prepared by transferring the mats from the agar surface into flasks containing glass beads and neopeptone-maltose broth, shaking vigorously for 10 minutes, and then straining through several layers of cheese-cloth. The spores were counted on a hemocytometer and the suspensions diluted with broth to contain approximately 50000000 spores per cc. Each serial dilution-tube received 0.1 cc of the adjusted spore suspension. Incubation was carried out at 28°C for 14 days. Dilutions showing complete inhibition of growth were tested for fungicidal action by subculturing into 10 cc of the test broth plus serum and incubating at 28°C for at least 14 days.

Results. Antifungal response to each of the agents tested by the agar cup-plate method is shown in Table I. It is recognized that this method does not always give a true expression of antifungal activity, since inhibition will depend largely upon penetration of the test substance through the medium. The data are presented, however, to indicate the close agreement in the order of activity obtained with this procedure and the serial dilution technic (Table II). As shown by both methods, polymyxins B and E were the most active members of the antibiotic group, appearing equally effective against the respective fungi tested.

In the presence of serum, polymyxins B and E proved equal to G-4 in fungicidal activity against *Candida albicans,* while benzoic acid, salicylic acid, undecylenic acid and Asterol were ineffective at the maximum concentration employed (Table II). G-4 was the most active agent against *Trichophyton mentagrophytes;* undecylenic acid and Asterol were approximately twice as effective as polymyxins B and E, while benzoic acid and salicylic acid failed to inhibit this organism at 25 mg %. Against *Trichophyton purpureum,* polymyxin E was again more active than the organic acids in the presence of serum. The fungicidal activity of polymyxin E was also greater than that of benzoic acid and salicylic acid against *Microsporum audouini,* equal to that of undecylenic acid, but less than that of Asterol and G-4.

Antifungal activity of the polymyxins did not appear to be significantly antagonized by serum. Benzoic acid and salicylic acid were only slightly affected, while activity of undecylenic acid and G-4 was markedly decreased. The presence of protein seemed to increase Asterol activity. Of interest in this regard was a similar observation(10) of an enhancing effect of agar upon this compound.

Discussion. Polymyxins B and E appear to possess potentialities as antifungal agents. Based on *in vitro* tests, these antibiotics might possibly be more effective than some antifungal drugs in current use. The absence of sensitizing properties of polymyxin B(11), for example, would make it ideally suited for topical application. Polymyxins B and E were the most active of the substances tested against the systemic fungus *Debaryomyces neoformans* in the presence of serum (Table I). Recent reports(12-14) favoring the parenteral use of polymyxin B for certain bacterial infections would suggest trial of this antibiotic for therapy of various systemic mycoses.

It has been the experience of clinicians that the use of antibiotics may result in overgrowth of organisms not susceptible to their action, *e.g.,* Monilia and certain species of Gram-negative bacteria. In view of the susceptibility of *Candida albicans* to polymyxins B and E and the marked antibacterial activity they possess against Gram-negative organisms,

TABLE II. Fungicidal Activity of Polymyxins and Various Known Agents.

Agent	Minimum fungicidal concentration in mg %								
	C. albicans		*T. mentagrophytes*		*T. purpureum*		*M. audouini*		*M. lanosum*
	S*	NS†	S	NS	S	NS	S	NS	NS
Polymyxin A	>25	>25	>25	25					
B	25	25	25	25					25
D	>25	>25	>25	>25					
E	25	25	25	25	25	25	12.5	25	
Benzoic acid	>25	>25	>25	>25	>25	25	25	12.5	25
Salicylic acid	>25	>25	>25	>25	>25	25	>25	25	25
Undecylenic acid	>25	25	12.5	6.25	>25	6.25	12.5	3.12	3.12
Asterol	>25	>25	12.5	>25	3.12	>25	6.25	25	>25
G-4	25	3.12	6.25	.78	6.25	.78	6.25	.39	.78

* Medium plus serum. † Plain medium.

it would appear unlikely that overgrowth of such types would be encountered during systemic use of these antibiotics. However, there would be the possibility that an increase in Gram-positive flora might result.

Summary. (1) Polymyxins A, B, D and E were tested for antifungal activity against 5 species of dermatophytes and 1 systemic fungus. Varieties B and E were the most active and of equal effectiveness. (2) Activity of the polymyxins was not significantly inhibited by serum. (3) Clinical trial of the topical and systemic efficacy of polymyxins B or E in mycotic infections is suggested.

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Terramycin Hydrochloride Alone and with Mapharsen and Bismuth in Treatment of Experimental Syphilis of Rabbits. (19301)

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As shown by Hobby and her associates(1), terramycin hydrochloride is absorbed readily from the gastrointestinal tract of rabbits with high concentrations in the blood within an hour following the oral administration of the compound. Under the circumstances we have thought it advisable to determine the therapeutic activity of terramycin hydrochloride alone and in combination with Mapharsen and bismuth salicylate in the treatment of acute

syphilitic orchitis of rabbits although Hendricks and his colleagues(2), and Schoch and Alexander(3) have found the compound by oral administration therapeutically effective in the treatment of early syphilis of human beings.

Methods and materials. Rabbits were inoculated intratesticularly with the Nichols-Hough strain of *T. pallidum* and treatment instituted 4 to 5 weeks later in animals developing acute orchitis with positive darkfield examinations. Following the institution of treatment, clinical and darkfield examinations were made daily for 3 days in succession and thereafter at weekly intervals over a period of 70 days at which time lymph nodes were inoculated intratesticularly in rabbits. These animals were kept under observation with repeated darkfield examinations, for a period of 8 weeks when the results were evaluated. Terramycin hydrochloride* was administered orally in sterile 5% solutions of acacia. Also by intramuscular injection in the same vehicle. Suspensions in sterile peanut oil could not be administered because of gelling. Rabbits with acute syphilitic orchitis were also treated with terramycin hydrochloride by oral and intramuscular administration along with Mapharsen (oxophenarsine hydrochloride) by intravenous injection and bismuth salicylate in oil with Chloretone (Parke, Davis & Co.) by intramuscular injection, for possible synergistic or additive chemotherapeutic activity. Two rabbits were treated with the same dose or doses in all treatment schedules.

Results. Oral administration. Single doses of 50, 75, and 100 mg per kg of weight of terramycin hydrochloride were without demonstrable treponemicidal activity. Single doses of 150, 200, and 250 mg per kg showed temporary treponemicidal effects but were not completely curative since the results of lymph node transfers were positive in all animals. Much better results, however, were observed when the compound was administered orally twice daily in doses of 2.5, 5, 10, and 15 mg per kg of weight for 15 days in succession. In these experiments the total minimal curative dose with negative lymph node transfers was approximately 150 to 300 mg per kg of weight. The single doses were well borne with no evidences of toxicity. The same was true of rabbits given 2.5 and 5 mg per kg twice daily over a period of 15 days. Doses of 10 and 15 mg per kg twice daily for 15 days in succession, however, produced anorexia with progressive loss of weight. Thus one of 2 rabbits given 15 mg twice daily for 15 days (totalling 450 mg) died on the 40th day while each of 2 rabbits given 20 mg twice daily for 15 days (totalling 600 mg) died 4 days after the cessation of treatment.

Intramuscular administration. Much better treponemicidal effects were observed when terramycin hydrochloride was given by intramuscular injection in single doses of 25, 50, 75, 100, 150, and 200 mg per kg of weight. Single doses of 25 and 50 mg produced temporary negative darkfield examinations but were not completely curative since positive lymph node transfers were observed. According to our results the single minimal curative dose by this route of administration was approximately 75 to 100 mg per kg of weight. When administered once a day in dose of 2.5, 5, 10, and 25 mg per kg of weight for 15 days in succession, the total minimal curative dose was approximately 75 to 100 mg per kg of weight. All rabbits given single intramuscular doses of 25 to 200 mg per kg of weight survived with no evidences of general toxic effects. The same was true of all rabbits given 2.5 to 25 mg per kg once daily for 15 days in succession, totalling 37.5 to 375 mg per kg of weight. On the other hand, however, inflammatory reactions were generally observed at the sites of injection which progressed to necrosis in some animals given the larger doses.

Terramycin hydrochloride in combination with Mapharsen and bismuth. The oral administration of 2.5 mg terramycin hydrochloride per kg of weight twice daily for 15 days in succession (totalling 75 mg) was without detectable therapeutic activity. However, when given along with Mapharsen by intravenous injection in dose of 0.3 mg per kg every 3 days for 5 doses (totalling 1.5 mg), this dose of terramycin hydrochloride was

* Kindly supplied by Chas. Pfizer and Co., through the courtesy of Dr. Ray A. Patelski.

completely curative with negative lymph node transfers. The same results were observed when 0.5 mg bismuth salicylate per kg of weight was given every 3 days for 5 doses (totalling 2.5 mg) by intramuscular injection. Similar results were observed with terramycin hydrochloride administered intramuscularly. Thus the administration of 2.5 mg per kg once daily for 15 doses (totalling 37.5 mg) was without detectable therapeutic activity. But when administered along with 0.3 mg Mapharsen per kg by intravenous injection every 3 days for 5 doses (totalling 1.5 mg), this dosage of terramycin hydrochloride was completely curative with negative lymph node transfers. The same results were observed when bismuth salicylate was administered intramuscularly every 3 days for 5 injections in dose of 0.5 mg per kg (totalling 2.5 mg). Since both Mapharsen and bismuth salicylate alone in these doses per kg of weight were not completely curative with positive lymph node transfers, it is apparent that both compounds act synergistically or additively with terramycin hydrochloride in the treatment of experimental syphilis of rabbits, as has been observed with penicillin (4-6).

Summary. (1) Terramycin hydrochloride by oral administration in single doses of 50 to 250 mg per kg of weight were not completely curative in the treatment of acute syphilitic orchitis of rabbits. (2) When administered orally twice daily for 15 days in succession the total minimal curative dose was 150 to 300 mg per kg of weight. (3) By intramuscular injection the single minimal curative dose was 75 to 100 mg per kg of weight. When administered once daily for 15 days in succession the total minimal curative dose was 75 to 150 mg per kg by this route of administration. (4) Mapharsen by intravenous injection and bismuth salicylate by intramuscular injection yielded pronounced synergistic or additive therapeutic effects when given along with terramycin hydrochloride by oral and intramuscular administration.

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Hibernation in the Alligator. (19302)

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In common with several other species of reptiles the reaction of the alligator to various types of stimuli is slow enough to allow the observer much more time than would be the case if warm blooded animals had been employed (1,2). Unfortunately the concentration of various blood constituents is not constant but is subject to certain seasonal changes. McIlhenny (3) commented on the fact that the alligator does not eat for several months in the winter either in his native habitat or in the confines of a heated zoological garden. This loss of the desire to eat constituted almost the sole experimental evidence that the alligator is an animal which hibernates in the winter. In the course of over 3 years of research we have been able to collect a considerable amount of data on the effect of season on the alligator and on the caiman, a closely related tropical species.

Effect of temperature and light on blood glucose and growth rate. About 200 alligators and 35 South American caimans were observed under laboratory conditions. Pre-

TABLE I. Effect of Length of Day on Growth Rate and Fasting Blood Glucose.

Hr light	Nov. 19	Dec. 20	Jan. 17	Feb. 21	Mar. 23	Apr. 20	June 10	July 5	Aug. 8	Sept. 12	Oct. 18	Nov. 8	Dec. 5
						Glucose, mg %*							
10	73	75	109	128	123	121	118	94	136	114	48	66	60
14	75	81	110	133	118	107	119	89	143	121	40	39	52
						Body wt in g*							
10	40	40	37	41	51	59	66	74	81	81	—	—	79
14	44	43	39	45	59	68	79	82	86	83	—	—	80

* Each number represents avg of 12 alligators.

liminary studies on the alligator revealed that hypoglycemia and anorexia were coincident with the approach of winter regardless of the temperature of the animal's habitat. To determine the exact effect of temperature and light on hibernation 24 three-month-old alligators were selected and divided into 2 groups of 12. Both groups were placed in a constant temperature room which had no windows but which was lighted by fluorescent lights. One group was exposed to the artificial light for 10 hours a day and the other group to the same light for 14 hours a day for a period of over a year. The room temperature was 28°C, which is the average temperature of New Orleans on June 21. The longest days in the locality are 14 hours in June and the shortest are 10 hours in December. Under these conditions both groups were kept at the summer temperature; one group was exposed to "December light" and the other group to "June light". The animals were weighed and measured at approximately monthly intervals and glucose was determined by the Folin-Wu method using 0.1 ml of blood from the tip of the tail. Skinned white rats were fed to both groups. From Table I, which presents the average body weights and blood glucose values for each group, it would appear that hypoglycemia began in both groups in October and ended in late January. Differences between the two groups are probably not significant. It is curious that animals maintained under these constant conditions should still exhibit the usual seasonal hypoglycemia. Whatever mechanism is involved it seems to be independent of light or temperature. Both during the course of the light experiment and for the next 2 years blood glucose was determined on scores of other alligators at all seasons of the year. The results confirmed the observation that the glucose level drops in October or November and rises again in February or March. Blood glucose analyses were also performed each month on 30 caimans *(C. latirostris)*. There is some doubt as to the exact point of origin of these animals although it is known that they came from somewhere in South America. No significant variation in monthly blood glucose has been observed in the eight months they have been kept in the laboratory; the highest blood glucose was 94 mg % and the lowest was 76 mg %. There is no reason to believe that the caimans, which are tropical reptiles, hibernate in the winter months since their blood glucose level seems to be reasonably constant and since they ate as well in January as they did in July.

Effect of semi-starvation on seasonal blood glucose. Nine 2-year-old alligators were fasted from Dec. 19 to May 9 and frequent glucose analyses were conducted to see if semi-starvation would prevent the rise in blood glucose in the spring. The average blood glucose did not rise as long as the animals were fasted although the alligators showed obvious signs of hunger when any food was placed near them. It would seem that the anorexia is not necessarily associated with hypoglycemia although the two occur at the same time in the normal seasonal cycle. In spite of the fact that the animals were seriously emaciated on May 9, the last day of the fast, the glucose rose to the normal seasonal level after 2 weeks of feeding and the alligators quickly regained their lost weight.

Effect of hormones on fasting blood glucose. Thirty small alligators were divided into 3 equal groups in December. One group re-

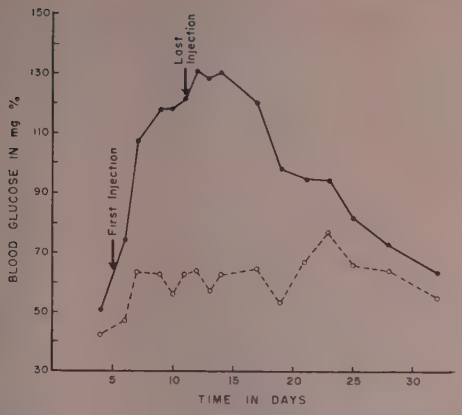

FIG. 1. The effect of whole sheep pituitary powder (Armour) on the fasting blood glucose of the alligator (December). Solid line represents avg of 5 experimentals injected daily with .3 mg/100 g body wt; the broken line represents avg of 4 controls.

ceived 0.1 mg desiccated thyroid per 100 g body weight intraperitoneally every other day; another group received 5 mg/100 g body weight of whole sheep pituitary powder (Armour) every other day, and the third group served as controls. After 2 weeks of these injections the average glucose level of the thyroid series was 35 mg %, the average of the 6 survivors of the pituitary injections was 90 mg %, and the average of the controls was 33 mg %. The pituitary series was discontinued and although the thyroid series was continued for an additional 3 weeks the blood glucose level at the end of that time was still no higher than that of the control group. Further studies of the action of crude pituitary powder were conducted in January on nine 18 inch alligators. Five of these animals received 0.3 mg whole pituitary powder per 100 g of body weight each day for 6 days while the other 4 served as controls. The results are recorded in Fig. 1. Successive injections of powdered desiccated liver or brain suspensions had no effect on the level of glucose of the controls. Although it would appear that the alligator is quite sensitive to very small amounts of whole pituitary powder it is by no means certain that the variations in the output of a "glycotropic" factor from the animal's own hypophysis are responsible for the seasonal rise and fall in blood glucose.

Effect of season on oxygen consumption and metabolic rate. It was considered possible that the failure of the alligators to eat for a large part of the year could be due to a hormone which controls metabolism and which directly or indirectly controls the desire to eat. Experiments were devised to measure the oxygen consumption in both winter and summer and to attempt to relate it to the metabolism. The measurement of oxygen consumption was accomplished by the relatively simple method of measuring the decline in pressure of the air in a calibrated vacuum desiccator which contained the alligator and wet soda lime to absorb the CO_2. All measurements were performed at the average summer temperature for New Orleans (28°C) by submerging the desiccator in a water bath or by placing it in a large temperature controlled incubator. All animals were allowed to equilibrate with the temperature at 28° for several hours and to accustom themselves to their temporary surroundings before measurements were taken. The smallest animals were allowed to remain in the desiccators for periods of about a day for each run. At the close of each experiment the air in the desiccator was flushed out by use of a water pump, the alligator was again equilibrated, and another run was begun. Three runs were performed on each small animal. The procedure was the same for larger alligators (up to 3 kg) except that the high rate of oxygen consumption necessitated a shorter test period which varied from about 1 hour for the very largest animals to 10 hours for some of 300 g size. In most cases 5 or 6 separate oxygen consumption tests were run on the larger animals. Since it is never possible to eliminate entirely the increase in oxygen consumption occasioned by movement of the test animal it was decided to use only the run in which the animal consumed the least oxygen in a given time. In most cases the first run gave results which were considerably higher than the second while the second and third runs usually checked to within about 10%. It must be emphasized that none of the results obtained were considered to be indicative of

TABLE II. Effect of Season on Oxygen Consumption by 19 Alligators and 17 Caimans.

	Alligator avg		Caiman avg	
	Winter	Summer	Winter	Summer
Body wt	49.7	87.7	116	161
M1, O_2/g/day	1.92 ± .07*	1.77 ± .06	1.69 ± .08	1.53 ± .07

* P.E. $= .6745 \sqrt{\frac{(v)^2}{n(n-1)}}$.

"basal metabolism" since that state would be difficult to define in a crocodilian. Under the conditions of these experiments the animals usually remained very quiet and in the majority of cases hours would pass without the slightest sign of motion. The winter series of experiments was conducted in December and January and the summer series in June and July. All animals were fasted for at least 3 days prior to the run which was finally recorded. Since an increase in body weight between the winter and summer experiments led to an increase in surface area of unknown magnitude it seems reasonable to equate the oxygen consumption in terms of ml O_2/g/24 hours. It was possible to demonstrate that the largest animals (3 kg) used about 0.5 ml O_2/g/day while the smallest (about 50 g) consumed about 2 ml/g/day.

The results of seasonal metabolic studies on one group of small alligators and on one group of caimans appear in Table II. It can be seen that the oxygen consumption per unit weight is slightly less in summer than in winter in both alligators and caimans. This probably does not indicate a lower "absolute" metabolic rate but rather a decrease in apparent metabolic rate due to decreased surface area per unit body weight. It is perhaps natural to expect closely related species such as the alligator and caiman to have the same metabolic rate and all evidence points to this as being correct.

Effect of hormones on metabolic rate. Since hypoglycemia is the most marked feature of hibernation in the alligator it was decided to determine the effect on metabolism of various substances which might change the blood glucose concentration. Five fasting alligators with an average weight of about 100 g were selected for study. Each animal was placed in a desiccator and control runs were performed on each for 3 successive days. The animals were then removed from the desiccators and placed in a tank of water for several hours to repair any effects of dehydration. Each was then injected intramuscularly with regular insulin (5 units/kg) and again placed in his respective desiccator. Since insulin does not produce any hypoglycemia for about 7 hours (unpublished observations) the animals were allowed to equilibrate for about 5 hours before the desiccators were sealed. In view of the fact that insulin in the dose used in this experiment exerts an effect for about 48 hours, only 2 separate 24-hour runs were conducted. Following the disappearance of the insulin from the blood each animal received 500 mg/kilo of glucose intraperitoneally and the oxygen consumption was measured on each of the next 2 days. After the disappearance of the glucose each animal was again removed, given water to drink, and then injected with 5 mg/100 g of crude whole pituitary powder (Armour). Twenty-four hours later each was again injected with 10 mg/100 g and oxygen consumption was measured on each of the next 2 days. The results are presented in Table III. Pituitary powder produced a slight but possibly significant increase in the rate of oxygen consumption whereas glucose and insulin seemed to have no effect. Since the significance of the results of the short term injections of pituitary powder on metabolism was questionable, 25 other alligators were selected for experiments to determine the effect of prolonged injections of desiccated thyroid and pituitary powder. Ten animals received 0.1 mg of desiccated thyroid every other day for 3 weeks, 10 received 5 mg of whole pituitary powder every other day for 2 weeks and 5 animals served

TABLE III. Influence of Insulin, Glucose, and Pituitary Powder on Oxygen Consumption (ml/g/day) on 5 Alligators.

	Body wt, g	Control	Insulin	Glucose	Pituitary powder
	114	1.08	1.29	1.32	1.33
	107	.96	1.11	1.18	1.24
	170	1.49	1.31	.94	1.22
	92	1.37	1.41	1.38	1.54
	73	1.90	1.88	1.97	2.56
Avg	111	1.36	1.40	1.36	1.58

TABLE IV. Influence of Prolonged Administration of Desiccated Thyroid and Pituitary Powder on Oxygen Consumption.

	No. alligators	Avg body wt	Ml O_2/g/day, avg and range
Control	5	42	1.73 (1.26–2.00)
Thyroid	10	42	1.83 (1.06–2.38)
Pit. powder	5	45	2.20 (1.83–2.70)

as controls. Unfortunately the pituitary powder was toxic enough to kill 5 of the 10 in that series before the oxygen studies were conducted. At the conclusion of the periods of injection oxygen consumption was measured on each of the alligators for 3 successive days. To make the experiment more nearly comparable to the one presented in Table III the lowest oxygen consumption of the first 2 runs was used to compute the averages shown in Table IV. Again it appears that pituitary powder produces an increase in metabolism in the alligator. It is questionable whether thyroid powder in the quantity injected is instrumental in producing much change in the metabolic rate in spite of the fact that the dosage employed is several times that required to produce an effect in a mammal.

Summary. Although alligators are quite active in the winter if the temperature is high they have a pronounced hypoglycemia and no appetite. There is no seasonal change in metabolic rate in either the alligator or caiman. The caiman does not hibernate. Pituitary powder produces prolonged hyperglycemia and increases the metabolic rate. Desiccated thyroid powder has no effect on blood sugar. Neither glucose nor insulin had any effect on metabolic rate.

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Effect of Parahydroxypropiophenone on Experimental Ovarian Tumors in Rats.* (19303)

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Experimental gonadal tumors have been developed in this laboratory by transplantation of a gonad into the spleen of castrated animals(1,2). Since it is postulated that the pituitary of such an animal is subjected to decreased gonadotrophin inhibition, it would follow that excessive or uninhibited pituitary gonadotrophin production is probably a factor of prime importance in the formation of these tumors. The fact that exogenous gonadotrophins will accelerate such tumor formation(3), lends credence to the validity of this hypothesis.

The present work was undertaken to investigate the property of non-estrogenic pituitary inhibition(4,5) attributed to parahydroxypro-

* This investigation was supported in part by the Damon Runyon Memorial Fund.

TABLE I. Twelve Female Castrate Rats With an Ovary Transplanted to the Spleen Fed 100 mg Parahydroxypropiophenone Daily.*

Days	Wt (g) Initial	Wt (g) Final	Size of transplant, mm
13	139	135	2 × 1
20	142	149	1
42	139	151	1
50	158	184	—
91	232	225	4
101	192	184	5 × 3
125	175	200	2 × 1
130	157	183	1 × 2
140	196	210	2 × ½
160	186	190	5 × 3
183	177	190	2 × 2
200	192	240	1

* All rats showed uterus with hyperplasia and/or squamous metaplasia of endometrium, and vagina showed cornification.

TABLE II. Five Female Castrate Rats With an Ovary Transplanted to the Spleen Fed 100 mg Parahydroxypropiophenone and 18 mg Thyroid Daily. See footnote Table I.

Days	Wt (g) Initial	Wt (g) Final	Size of transplant, mm
10	134	152	1
20	170	172	2 × 3
30	177	193	3 × 3
52	141	—	2
80	152	144	1

piophenone† since it was felt that such a substance should suppress the formation of the experimental ovarian tumors by inhibiting the production of pituitary gonadotrophins.

Method. Twenty-seven young adult female rats of the Long-Evans strain were given 100 mg daily of parahydroxypropiophenone in their diet, which consisted of Purina chow and water *ad libitum*. They were divided into 4 groups: (A) 12 castrated animals in which one ovary was transplanted to the spleen; (B) 5 castrated animals with one ovary transplanted to the spleen, which, in addition, received 18 mg daily of thyroid extract in their diet; (C) 6 castrated animals; and (D) 4 intact animals. Since previous reports claimed that parahydroxypropiophenone inhibited thyrotrophin(4), Group B was included to negate any changes that could be attributed to thyroid deficiency. The rats were sacrificed at intervals varying from 13 to 200 days, and complete autopsies were performed. In addition to the animals listed in Tables I and II there were 11 other animals in which adhesions developed from the spleen to the systemic circulation and these served as controls.

† Parahydroxypropiophenone was generously supplied by White Laboratories.

Results. In the intact animals vaginal smears showed that they entered into an estrus phase in 5 days and remained in that state for the experimental 90-day period. In all the animals in this experiment histologic examination of the uterus and vagina showed estrogenic stimulation characterized by hyperplasia and squamous metaplasia of the endometrium of the uterus, or severe vaginal epithelial cornification. (See Tables I-III). The ovarian transplants were dwarfed in size, none grew larger than 5 x 3 mm, and they were composed of small clusters of developing follicles with an occasional corpus luteum. There was no evidence of proliferation of the corpora lutea and no suggestion of a luteoma. This description applies to both Groups A and B. The 16 unlisted animals with splenic adhesions showed inhibited growth of the transplant and estrogenic stimulation of the uterus and vagina as described previously(2). Histologic examination of the thyroid gland showed a normal structure in each of the 5 groups.

Discussion. From previous reports(1,2) it has been shown that when an ovary is transplanted to the spleen of a castrated rat a series of proliferative changes occur characterized by the formation of corpora lutea which do not involute but proceed to form a luteoma and eventually a granulosa cell tumor. Such changes may be inhibited either by the addition of exogenous estrogen(6), or when adhesions occur that would circumvent the liver and hence allow estrogen to circulate systemically. In the present experiment the

TABLE III. Female Castrate Rats Fed 100 mg Parahydroxypropiophenone Daily. See footnote Table I.

Days	Wt (g) Initial	Wt (g) Final
17	196	189
55	195	160
55	228	160
62	212	205
70	171	180
76	173	185

transplanted ovary resembled in size and histologic pattern that seen in those castrate animals that develop adhesions or receive estrogen. As noted in our experiments, not only did the transplants behave as if they were under estrogenic stimulation, but also the uterus and vagina in the castrated Group C had the histologic pattern that results from estrogenic stimulation, which indicates that parahydroxypropiophenone apparently exerted its influence by its estrogenic activity. Group B with added thyroid behaved identically to Group A.

Conclusion. Parahydroxypropiophenone, when fed to castrate animals at a level of 100 mg a day, behaved like an estrogen by producing cornification of the vagina and hyperplasia and metaplasia of the uterine endometrium. Exogenous thyroid had no added effect other than that produced by parahydroxypropiophenone alone. The evolution of experimental ovarian tumors obtained by transplantation of an ovary to the spleen of a castrated rat was inhibited by parahydroxypropiophenone similarly to that reported when estrogens were given or when splenic adhesions circumventing the portal circulation were present.

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Resistance Induced by Alien Strain Mouse Lymphoid Tissue to Lymphosarcoma 6-C3H-ED in C3H Mice. (19304)

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The growth of transplanted tumors as influenced by the previous implantation of tumor cells or other tissues from alien strains of mice or rats has been the subject of much investigation. The literature up to 1929 has been reviewed by Woglom(1). Eisen and Woglom(2) reported protection of rats of strain 990 against the August carcinoma by implantations of embryo tissue from August strain rats, and Sturm(3) showed protection of Wistar rats against transplantable lymphatic leukemia by implantation of embryo or injection of blood from Wistar rats or embryo from Rockefeller Institute hooded rats. Protection of pure strain mice against growth of strain specific tumors (with the exception of certain leukemias) by such procedures has not been reported. Lewis(4) has shown that spindle cell sarcomas arising in inbred mice would grow progressively and kill when implanted in mice of the strain in which they originated. However, when transplanted in mice of alien genetic constitution the tumors regressed after initial growth. The sojourn of such tumors in alien strain mice conferred immunity against subsequent implantation of the same tumor. Implantation of various organs of alien strain mice had no inhibiting effect on the growth of the strain specific tumors. Data of similar significance were presented by Barrett(5). However, MacDowell, *et al.*(6) by prior implantation of embryo tissue from foreign strain mice, found that C58 mice were protected against strain specific transplantable leukemia, and Rhoads and Miller(7) similarly found that AR mice are protected against transplantable leukemia by courses of injections of emulsions of spleen and lymph node cells from the Rockefeller Institute strain of mice given prior to tumor cell implantation.

It was found during the course of studies on the progressive growth of lymphosarcoma 6-C3H-ED in CF1 mice treated with cortisone

acetate(8) that not only are CF1 mice in which the tumor had regressed immune, but similar immunity is conferred by the implantation of lymph node or thymus tissue from C3H mice. This observation led to attempts to protect C3H mice by the implantation of lymphoid tissue from CF1 mice. The present report is concerned with these results and those obtained using other tissues from CF1 mice, and from other random bred and pure line strains of mice.

Materials and methods. The Gardner lymphosarcoma 6-C3H-ED obtained from the Jackson Memorial Laboratory and carried in JAX C3H mice was used for implantation in all experiments. Implantation of the tumor was carried out by subcutaneous injection into the right axillary region of 0.1 ml of a cell suspension obtained by withdrawing the top portion from a suspension of tumor cells minced in saline. Counts indicated that the dose implanted contained approximately 5 million tumor cells. C3H mice so treated regularly die with enormous tumors within 18 to 22 days following implantation. Implantation of lymphoid tissues from donor mice was carried out using 18-gauge trocars loaded with one axillary and one inguinal lymph node and a ¼ portion of thymus. This tissue mixture was discharged subcutaneously into the left axillary region of recipient C3H mice. Foreign strain tumors (EO771 carried in C57 black mice, and lymphoid leukemia P1534 carried in DBA-2 mice), and other tissues were implanted in the same way. In most experiments spleen cell suspensions in saline were implanted by injection. 140 to 160 mg of spleen tissue was minced with scissors in 2.25 ml of saline and worked until it would pass through an 18-gauge needle. 0.25 ml volumes were injected into recipient mice. Specified tissues from various strains of mice were implanted subcutaneously in young adult 18 to 22 g C3H mice. After an interval (in most experiments 5 to 7 days) these mice were challenged on the opposite flank with 0.1 ml of a saline suspension of tumor cells. The mice were palpated at 2- or 3-day intervals, beginning on the fifth day following tumor cell implantation to determine growth of the tumor. Experiments were terminated 7 to 10 days after all tumor bearing mice had died. All survivors were then again challenged with lymphosarcoma cells to determine whether they were immune. C3H, DBA-2, C57 Black and CAF1 mice were obtained from the Jackson Memorial Laboratory. ZBC mice (produced by mating mice of the A strain and C3H (called Z strain) to produce F1 hybrids, and mating F1 females with Z males), were obtained from the surplus stock of Dr. J. J. Bittner, University of Minnesota. Random bred mice, CF1 and CFW, were obtained from Carworth Farms, and Manor mice were obtained from Manor Farms. Embryo tissue was obtained from pregnant CF1 females near term and mammary tissue from nursing CF1 dams. Male C3H mice were used in most experiments, but similar results have been obtained in the few experiments made with females.

Results. It was shown in repeated experiments that a significant number of C3H mice are protected against growth of lymphosarcoma 6-C3H-ED as a result of previous implantation of lymph node-thymus tissue from CF1 mice. It was of interest to determine whether the ability to protect against this tumor was peculiar to lymphoid tissue of CF1 mice, or whether other CF1 tissue would elicit this response. Tissue from other strains of mice were also studied as the work progressed. The results are tabulated in Table I.

It was found that lymph node-thymus mixtures from CF1 mice implanted in C3H mice 4 to 21 days prior to challenge with lymphosarcoma protected 119 (63%) of the 188 mice so treated. From 60 to 70% of mice implanted at intervals between these two extremes were protected in various experiments. This protection was manifest either by failure of the tumor to grow at the site of implantation or by regression of well defined palpable tumors. When the interval between tissue implantation and tumor cell challenge was reduced to 2 days, only 3 of 10 mice were protected, and none of the mice were protected which received the tissue on the same day, or 2 or 4 days after the day of tumor cell implantation. Spleen tissue of CF1 mice implanted either by trocar or as cell suspension protected 9 of 25 mice used

TABLE I. Growth of Lymphosarcoma 6-C3H-ED in C3H Mice as Influenced by Implantation of Tissues from Alien Strain Mice.

Species of tissue donor	Tissue implanted	Interval between tissue and tumor implantation, days	No. of mice in tests	Tumors failed to develop	Palpable tumors regressed	Progressive tumors	No. of mice protected	% protected
CF_1	Lymph node and thymus*	4-21 before tumor implantation	188	51	68	69	119	63
		2 " " "	10		3	7	3	30
		Same day	8			8		
		2-4 after tumor implantation	16			16		
C57 black		7 before " "	8			8		
DBA-2		"	6			6		
ZBC		"	8		2	6	2	25
CAF_1		"	8			8		
A		"	8			8		
Manor		"	11		6	5	6	54
CFW		"	8	1	2	5	3	37
C3H		"	10			10		
C57 black	Mammary carcinoma EO771*	"	8			8		
DBA-2	Lymphoid leukemia*	"	8			8		
CF_1	Embryo*	"	10			10		
	Leg muscle*	"	18			18		
	Heart muscle*	"	10			10		
	Mammary tissue*	"	8			8		
	Spleen†	5 "	8		3	5	3	37
	" *	7 "	10		2	8	2	20
	" *	"	7	2	2	3	4	57
ZBC	" †	"	15	13	2		15	100
	" †	5 "	8	7		1	7	87
	" *	7 "	7	6	1		7	100
Manor	" †	5 "	8		3	5	3	37
DBA-2	" †	"	8		2	6	2	25
C57 black	" †	"	8		2	6	2	25
CFW	" †	"	8		4	4	4	50
CAF_1	" †	"	8			8		
A	" †	"	8			8		
C3H	" †	"	8			8		
Untreated controls			112			112		

* Implanted by trocar. † Implanted as cell suspension.

in 3 experiments, but embryo skin, leg muscle, heart muscle, and mammary tissue from CF1 mice were without effect. Neither spleen nor lymph node-thymus mixture from CAF1, A or C3H mice protected C3H mice. Lymph node-thymus mixtures from C57 and DBA-2 mice failed to protect, but spleen tissue from both strains protected 2 of 8 mice. Both spleen and lymph node-thymus mixtures from Manor, CFW and ZBC mice gave protection. The results obtained with ZBC spleen are of particular interest; 29 of 30 mice treated in 3 experiments were protected. Mammary carcinoma EO771 growing in C57 mice, and lymphoid leukemia P1534 growing as a subcutaneous tumor mass in DBA-2 mice, when transplanted to C3H mice failed to protect against lymphosarcoma 6-C3H-ED subsequently implanted. Spleen or lymph node-thymus tissue from C3H mice implanted in C3H mice failed to protect. All of 112 untreated C3H control mice implanted with lymphosarcoma died of progressive tumor growth.

The high incidence of regression of palpable tumors in treated mice is of particular interest. The regressions observed followed a definite pattern with respect to time of occurrence. In mice implanted with spleen or lymphoid tissue 5 or 7 days prior to tumor cell implantation, rapid disappearance of large masses (1000-3000 mm^3) occurred between the fourteenth and sixteenth day after tumor implantation. All of the mice in which regression occurred, as well as those in which no tumor developed, were found to be immune upon challenge with lymphosarcoma cells 30 to 35 days following the original tumor implantation.

In experiments not recorded it was shown that such lymphosarcoma immune mice were not immune to Sarcoma 180 or Mammary Carcinoma C3H-BA.

Discussion. It is known that resistance to strain specific transplantable leukemia in inbred mice may be induced by injection of certain tissues from alien strain mice prior to tumor inoculation. The experiments reported in this paper establish the same fact for transplantable lymphosarcoma in C3H mice.

Lymph node-thymus or spleen tissue from CF1 mice implanted into C3H mice protect against progressive growth of lymphosarcoma 6-C3H-ED subsequently implanted. This property appears to be specific for these tissues since CF1 embryo, mammary gland, leg or heart muscle failed to induce resistance in similar experiments. In this respect our results differ from those of MacDowell *et al.* (6) who found C58 mice to be protected by implanting embryo tissue, but resemble those of Rhodes and Miller(7) who reported AR mice to be protected against transplantable leukemia by prior injections of lymph node and spleen mixtures from an alien strain.

The ability of spleen or lymphoid tissue to protect C3H mice against the C3H lymphosarcoma varied with the strain of donor mouse. Neither lymph node-thymus nor spleen of A or CAF1 mice protected. Lymph node-thymus from C57 and DBA-2 mice were inactive, while spleen from these strains protected a few mice in the various experiments. Both lymph node-thymus mixtures and spleen tissue from the random bred mice (CF1, CFW and Manor) were active. CF1 lymph node-thymus mixtures, and ZBC spleen tissue appeared to be the most active for inducing protection. ZBC spleen tissue was highly active; only 1 of 30 mice implanted with this tissue failed to resist lymphosarcoma growth on subsequent challenge. The reason for our failure to find more than partial protection of the mice in various experiments remains obscure, and we continue to seek the conditions necessary to increase the percentage of mice protected.

An interval of from 4 to 7 days between tissue implantation and subsequent tumor cell challenge appears to be necessary for significant protection. This is shown in the experiments done with CF1 thymus-lymph node implantation wherein over 60% of the mice implanted 4 or more days before tumor challenge were protected. Mice implanted with CF1 lymph node-thymus mixtures on the day of tumor implantation, or 2 or 4 days thereafter were not protected.

Summary. Lymph node and thymus mixtures, or spleen tissue from certain strains of mice implanted in JAX-C3H mice, 4-7 days

prior to subcutaneous injection of lymphosarcoma cells, protect some of the mice so treated against progressive growth of the tumor. This protection is manifest either by failure of the tumor to grow at the site of implantation or by regression of palpable tumors. Such protected mice are immune to subsequent reimplantation of the tumor. Mice implanted 2-4 days after, or on the day of tumor implantation are not protected. Embryo skin, mammary tissue, leg or heart muscle, from mice whose lymphoid tissue is active failed to protect. Under the conditions of the experiments tissue from some strains of mice were more powerful in eliciting protection than those of other strains.

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Retardation and Regression of Lymphosarcoma in C3H Mice Treated with Alien Mouse Spleen and A-Methopterin. (19305)

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It was shown in a previous publication(1) that lymph node-thymus mixtures, or spleen tissue from certain alien strains of mice, implanted into C3H mice induced a state of resistance to progressive growth of lymphosarcoma 6-C3H-ED subsequently implanted. This resistance was well developed in mice implanted 4 to 21 days prior to tumor implantation, and less so in mice implanted 2 days before, but was not evident in mice implanted with lymphoid tissue on the same day or 2 to 4 days following the day on which tumor cells were introduced. Spleen tissue from ZBC mice was found to be particularly active in eliciting the resistant state. It was postulated that advantage could be taken of this to treat C3H mice bearing lymphosarcoma if the growth of the tumor could be retarded long enough for the resistant state to develop. The present paper is concerned with experiments in which delay of tumor growth was attempted by using A-methopterin, and by use of small numbers of malignant cells to induce tumors.

Materials and methods. Lymphosarcoma 6-C3H-ED obtained from the Jackson Memorial Laboratory and carried in JAX-C3H mice was used in all experiments. This tumor arose in C3H mice, and in our experience grows progressively and kills all C3H mice implanted with it. Eighteen to 22 g C3H mice were employed as test subjects. The tumor was induced by subcutaneous injection of 0.1 ml volumes of saline suspension of minced tumor cells from 10- to 14-day-old tumors in C3H mice. Tumor cell dosage was determined by hemocytometer counts. Spleens removed from ZBC mice were used. (These mice are a backcross generation to the C3H strain of Dr. J. J. Bittner, University of Minnesota).* Several spleens were pooled and minced with scissors and suspended in saline. The mice received one 0.25 ml injection of spleen cell suspension through an 18-gauge needle into the left flank. The dose represented approximately 20 mg of moist spleen tissue. A-methopterin was administered sub-

* ZBC mice are produced by mating mice of the A strain and C3H (called Z strain) to produce F_1 hybrids. The F_1 females are crossed with Z males and the resulting animals are ZBC's.

cutaneously in the abdominal region. The mice were palpated daily beginning on the fifth day following tumor cell implantation, and observations were continued for a 45-day period. Additional details are given in connection with the various experiments.

Experimental. A-methopterin (4-amino-N-methyl folic acid) is known to retard the growth of lymphosarcoma 6-C3H-ED in C3H mice. As a preliminary step an experiment was set up to determine whether this compound influences the development of the resistance induced in C3H mice by implantation of ZBC spleen.

Two groups of 15 mice each were implanted subcutaneously in the left flank with ZBC spleen cell suspension. After 5 days these mice and 15 untreated controls were implanted on the right flank with approximately 5,000,000 lymphosarcoma cells. Drug treatment was begun on the day of tumor implantation as follows: Group 1, A-methopterin 2 mg/kg/day for 11 consecutive days; Group 2 received no drug; Group 3 served as untreated controls. Mice treated with spleen and A-methopterin failed to develop tumors, as did those treated with spleen alone. All untreated controls died with enormous tumors on the 21st to 23rd day.

The experiment showed that spleen cell induced resistance to lymphosarcoma developed in mice being treated with A-methopterin. An experiment was done in which 3 groups of C3H mice were implanted with different doses of lymphosarcoma cells 48 hours before starting treatments. One group (A) was implanted with 5,000,000, another (B) with 500,000, and the last (C) with 50,000 tumor cells. These 3 groups were then subdivided into 4 additional groups: one of which received A-methopterin, 2 mg/kg/day for 11 consecutive days; another group received one injection of ZBC spleen cell suspension; the third, one injection of ZBC spleen cell suspension, and A-methopterin 2 mg/kg/day for 11 consecutive days; the fourth group served as untreated controls. The results are shown in Experiment 1 in Table I. Other experiments were made in which groups of C3H mice were implanted with approximately 250,000 tumor cells; treatments were begun in one, 24 hours, and in the other 96 hours following tumor cell implantation. Results of this work are shown in Experiments 2 and 3 in Table I.

It is seen in Experiment 1 that among the mice implanted with 5,000,000 tumor cells 48 hours before treatments were begun, 3 of the 5 mice which survived the combination treatment with ZBC spleen and A-methopterin were protected as compared with 1 of 8 treated with A-methopterin alone. All of the ZBC spleen-treated mice and all controls died of progressive tumor growth. Of those mice implanted with 500,000 tumor cells and given the combination treatment 5 out of 8 were protected; of those treated with A-methopterin alone 2 of 8 were protected; one regression occurred among the 6 implanted with spleen tissue. Of those implanted with 50,000 tumor cells, all 5 mice receiving the combination treatment were protected, 2 out of 4 surviving the A-methopterin treatment were protected, and in 4 of the 5 receiving spleen implantation alone regression of palpable tumors occurred.

Untreated mice receiving larger numbers of tumor cells showed earlier appearance of tumors and died more quickly than those having received fewer cells. Those implanted with 5,000,000 tumor cells were palpable on the 5th day and all died on the 20th to 21st day; those implanted with 500,000 cells became palpable on the 7th day and died on the 24th day, and those implanted with 50,000 cells were palpable on the 10th day and all died on the 26th day. This experiment illustrates effective treatment of C3H mice with lymphosarcoma 6-C3H-ED.

In Experiment 2, similar results were obtained with mice in which treatment was delayed 24 hours after the implantation of 250,000 tumor cells. All 7 mice surviving the combination treatment with spleen cells and A-methopterin failed to develop palpable tumors; 6 of 8 treated with spleen cells alone were similarly protected; and 4 of 8 with treated A-methopterin alone were protected.

When mice were implanted with 250,000 tumor cells and treatment delayed for 4 days (Experiment 3), 5 of 8 mice treated with A-methopterin, and 4 of the 7 mice surviving the combination spleen cell and A-methopterin

TABLE I. Effect of Treatment with Alien Mouse Spleen, A-methopterin, and a Combination of Both on Growth of Lymphosarcoma 6-C3H-ED in C3H Mice.*

Experiment	Delay following tumor implantation before treatment was begun	No. of tumor cells implanted	Treatment	No. of mice in groups	No palpable tumors	Palpable tumors regressed	Progressive tumors	Died during treatment	No. of mice protected
1 (A)	48 hr	5000000	A-methopterin†	8		1 (23)	7		1
	,,	,,	ZBC spleen	8			8		
	,,	,,	A-methopterin† + ZBC spleen	8		3 (19)	2	3	3
	,,	,,	None	8			8		
(B)	48 hr	500000	A-methopterin†	8	2		6		2
	,,	,,	ZBC spleen	6		1 (17)	5		1
	,,	,,	A methopterin† + ZBC spleen	8	4	1 (35)	3		5
	,,	,,	None	8			8		
(C)	48 hr	50000	A-methopterin†	5		2 (31–35)	2	1	2
	,,	,,	ZBC spleen	5		4 (18–20)	1		4
	,,	,,	A-methopterin† + ZBC spleen	5	5				5
	,,	,,	None	5			5		
2	24 hr	250000	A-methopterin‡	8	3	1 (32)	4		4
	,,	,,	ZBC spleen	8	2	4 (16)	2		6
	,,	,,	A-methopterin‡ + ZBC spleen	8	7				7
	,,	,,	None	8			8		
3	96 hr	250000	A-methopterin§	8	3	2 (32–34)	3		5
	,,	,,	ZBC spleen	8			8		
	,,	,,	A-methopterin§ + ZBC spleen	8	2	2 (33–35)	3	1	4
	,,	,,	None	8			8		

* Tabulation 45 days after tumor implantation. † 2 mg/kg/day for 11 days. ‡ 2 mg/kg/day for 10 days. § 2 mg/kg/day for 7 days. ‖ Day on which regression was complete.

treatment were protected. The 8 control mice as well as the 8 receiving spleen cells alone all died of progressive tumor growth.

Discussion. The data show that C3H mice in which treatment was begun 24 or 48 hours subsequent to implantation of lymphosarcoma cells may be protected from progressive tumor growth by a combination treatment consisting of a single injection of spleen tissue and daily injection of A-methopterin. Treatment with either spleen cell suspension alone, or A-methopterin alone is less effective. When treatment is begun 4 days following implantation of the tumor, 5 of 8 mice treated with A-methopterin alone, and 4 of 7 mice (which survived combination treatment with A-methopterin and spleen cell suspension) were protected from progressive tumor growth. It would appear that under these conditions, tumor growth is retarded sufficiently to allow development of a state of resistance as a result of ZBC spleen cell implantation. This is shown by the greater number of protected mice among those treated with the combination than with either treatment alone (Experiments 1 and 2). Development of a resistant state is clearly indicated by the regression observed in mice implanted with 50,000 tumor cells and treated with a single implantation of ZBC spleen tissue in Experiment 1 and in those implanted with 250,000 tumor cells and similarly treated in Experiment 2.

The regressions observed in mice treated with A-methopterin alone, with the exception of one mouse (Experiment 1 (A)) all took place within 31 to 35 days following tumor cell implantation. This is in contrast to the regressions observed in mice treated with ZBC spleen cells alone (Experiment 1 and 2) which occurred 17 to 20 days after tumor cell implantation. In Experiment 3 (in which treatment was delayed 4 days) the regressions

observed took place 32 to 35 days after tumor cell implantation. The mechanism responsible for these regressions has not been determined and is the subject of further study.

Summary. Under certain conditions C3H mice implanted with lymphosarcoma 6-C3H-ED were protected either by injection of ZBC spleen tissue alone or by A-methopterin treatment alone. A more successful treatment consisted of a single subcutaneous injection of ZBC spleen tissue with daily doses of A-methopterin. Mice which had received tumor implantations 24, 48 or 96 hours preceding therapy were treated with this combination. Some were not protected, but in others tumors either failed to become palpable, or having become palpable appeared to have regressed completely.

ADDENDUM. The mice enumerated in the column, "Number of Mice Protected," shown in Table I, were challenged for immunity by injection of 5,000,000 lymphosarcoma cells subcutaneously into the left flank. The mice in Exp. 1 and 2 were challenged on the 42nd, and those in Exp. 3 on the 40th day of the experiment. Immune mice either developed small tumors which regressed, or no tumors at all; non-immune mice died within 30 days with progressive lymphosarcoma. Of the 14 mice which had been treated with A-methopterin alone in the three experiments, 6 were immune; of 11 which had been treated with ZBC spleen alone all were immune, while of the 24 which had received the combination treatment (A-methopterin and ZBC spleen) 20 were immune.

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Replacement of Protogen by Lipoic Acid in the Growth of *Tetrahymena*. (19306)

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The ciliate *Tetrahymena geleii* is readily grown in synthetic medium comprising, with only one exception, known chemicals. The one unknown factor is the liver fraction concentrate, protogen(1). Protogen has been identified(2) with the acetate(3) and the pyruvic oxidation(4) factors. Kidder and Dewey(5) found, as would thus be expected, that in *T. geleii*, protogen serves at least one other function than the replacement of acetate.

Reed and co-workers have recently(6,7) purified a series of factors from liver residues which show high activity in replacing both the acetate and pyruvic oxidation factors. One of these factors has been crystallized(8) and named, tentatively at least, lipoic acid. It was thus of interest to ascertain if this compound may replace protogen in the growth of *Tetrahymena geleii*.

Methods. *Tetrahymena geleii* S(9) was grown in the synthetic medium described by Elliott(10). Protogen was omitted from the medium and was replaced by varying amounts of lipoic acid.* Inoculations were made in all cases by loop transfer. Cells for inoculation into the first experimental culture were washed with 500 volumes of distilled water before transfer, to assure against a carry over of material. The data presented was obtained from cultures which had gone through three sub-cultures in the experimental medium. Growth was measured turbidimetrically after 96 hours growth at 25°C and is expressed as optical density.

Results and discussion. Fig. 1 shows that lipoic acid in concentrations of 1 unit†/ml and higher is capable of maintaining growth

* Protogen was generously supplied by Dr. E. L. R. Stokstad. Concentrates of lipoic acid were kindly supplied by Dr. L. J. Reed.

† One unit is equivalent to the manometric pyruvate oxidase response produced by 1 mg of yeast extract.

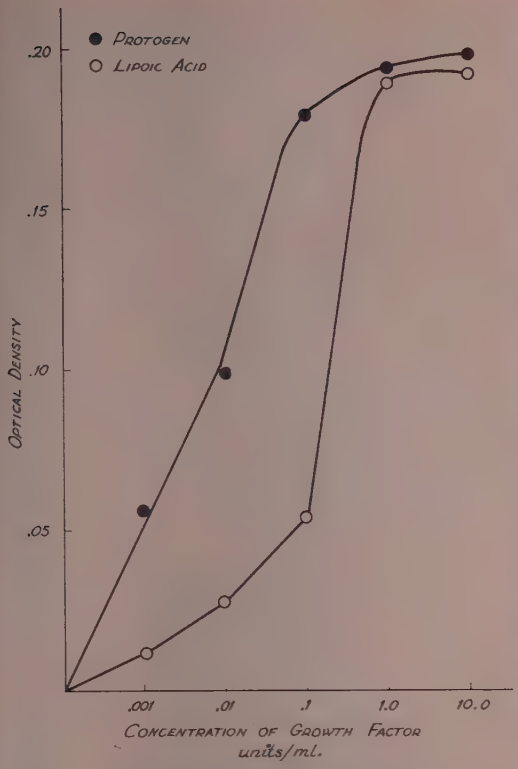

FIG. 1. Growth of *Tetrahymena geleii* S in response to various concentrations of protogen and of lipoic acid.

equal to that obtained in the presence of protogen. Since crystalline samples of neither protogen nor lipoic acid are available at present for investigation, it is of course not possible to designate one factor as more active than the other.

It has been suggested that lipoic acid may be a new B vitamin. It has been shown that there occur a variety of active forms of this factor in nature(6,7); this had been previously suggested by Snell and Broquist(2). Thus lipoic acid appears to resemble such vitamins as pyridoxine and niacin which also occur in nature in a variety of chemical forms. In fact, since lipoic acid replaces protogen, it appears that protogen may actually be either lipoic acid or one of its chemical derivatives.

Summary. Lipoic acid is capable of completely replacing protogen in the growth of *Tetrahymena geleii.*

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Use of Perchloric Acid for Nucleic Acid Histochemistry in Mammalian Nerve and Liver Cells.* (19307)

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Following the report of Ogur and Rosen(1) on the differential extraction of pentose nucleic (PNA) and desoxypentose nucleic acid (DNA) from plant and animal tissues with perchloric acid, there have been several attempts to apply this procedure to the histochemical demonstration of nucleic acid. Ogur and Rosen's procedure has been employed for this purpose on plant tissue(2), a proto-

* Partial accounts were presented at the Annual Meetings of the Histochemical Society March 1950, and 1951.

zoan organism(3), a bacterial species(4), and several mammalian species(5,6). The purpose of this paper is to present qualitative and quantitative data on the use of the perchloric acid extraction procedure in mammalian nervous tissue and liver.

Methods. The brain and spinal cord of cat, dog, rat and guinea pig and the liver of cat and dog, all fixed by vascular perfusion with a solution containing 10% formalin, 2.4% gum acacia and 0.9% sodium chloride(7), were employed in this study. Paraffin sections, 10 μ thick, were carried down to water and placed in 10% perchloric acid. Extraction was carried out at 4°C, 20°C and 37°C for varying periods of time to determine the minimum time necessary for the removal of PNA. Control sections were incubated in water at the same temperature and otherwise treated in an identical manner as the experimental sections. The affinity of nucleic acid for basic aniline dyes was employed to reveal PNA and DNA. Sections were stained with thionin buffered at pH 3.4(8). The Feulgen reaction, carried out according to the recommendations of Stowell(9), was used to reveal DNA. Quantitative determinations of a relative nature were made on the intensity of coloration of glial nuclei and liver cell nuclei with a direct photometric technic employing apparatus modified from that of Pollister and Moses (10). Green light provided by a Wratten filter No. 58 and a tungsten filament light source stabilized with a voltage regulator was used for illumination of the tissue sections. The Millon reaction, modified from Bensley's procedure(11) by carrying the procedure out at 56°C for 1½ hours, was used for the study of nerve cell protein. Photometric measurements of tyrosine mercurial were made at 3650Å by using a G. E. CH_4 lamp and a Corning filter No. 5840(12). The effect of perchloric acid treatment on cell structure was observed in unstained sections with the phase contrast microscope. Sections stained with the acid dyes, eosin and fast green, were also examined for structural alterations. The effect of the perchloric acid extraction procedure on non-specific light losses and specific absorption by tissue constituents other than PNA near the nucleic acid absorption maximum of 2600Å was studied with a direct photometric technic. For illumination a mercury resonance lamp (Hanovia) was used in conjunction with a quartz filter cell containing a solution of nickel sulfate and cobalt sulfate(13) to provide 2537Å radiation. The optical parts of the microscope consisted of a fused quartz condenser, a 50 X reflecting objective of N.A. .54 (American Optical Co.), and a 10 X quartz ocular. Tissue sections mounted in glycerin on quartz slides with quartz coverslips were focussed in visible light and were then ready for photometric measurements or photography in ultraviolet light without refocussing, because the reflecting objective is achromatic. Transverse sections, 10 μ thick, of cat skeletal muscle and spinal cord fixed as above were measured photometrically. The muscle fibres were found to have no cytoplasmic basophilia and were assumed to contain no PNA. These sections were photometered and photographed and the measurements repeated on the same sections after extraction in 10% perchloric acid for 15 minutes at 37°C. The sections of spinal cord were photometered untreated, then incubated in protease-free ribonuclease (0.1 mg/cc of McIlvain's buffer, pH 7.0 at 37°C for 3 hours) for the removal of PNA, photometered again, extracted with perchloric acid as for the skeletal muscle, and photometric measurements carried out the third time on the same section.

Results. Extraction of PNA. The procedure of Ogur and Rosen(1), namely, 18 hour extraction with 10% perchloric acid at 4°C, removed all cytoplasmic and nucleolar basophilia, which depends upon the presence of PNA, from liver cells but proved inadequate in achieving this goal in all the types of nerve cells examined. Even after 93 hours of extraction, considerable amounts of cytoplasmic and nucleolar basophilia remained in neurons. Higher temperatures were there-

TABLE I. Effect of Temperature on Time Required for Extraction of Cytoplasmic and Nucleolar Basophilia with 10% Perchloric Acid.

Temp., °C	Nerve cells	Liver cells
4	Incomplete at 93 hr	18 hr
20	12	3½
25	2	—
37	¼	<1/12

TABLE II. Effect of Perchloric Acid Extraction of Dog Liver and Cat Spinal Cord on Intensity of Feulgen Staining (Mean ± St. Dev. N = 10).

Cell nuclei	Condition of extraction °C	Time, hr	Extinction coef. control	Extinction coef. experimental	Change
Liver	4	18	.11 ± .02	.12 ± .02	0
		93	.15 ± .02	.15 ± .04	0
Astrocytes	20	16	.15 ± .01	.16 ± .01	0
		22	.13 ± .02	.05 ± .03	61.5% reduction Prob. <.01
	37	¼	.30 ± .02*	.29 ± .03*	0
		⅓	.18 ± .01	.15 ± .03	17.5% reduction Prob. <.05

* Central core of nucleus 2 in diam. measured as compared with 3.3 μ diam. core of nuclei for the other measurements.

fore employed for the extraction of nervous tissue. The results of this phase of the study are summarized in Table I. Sections from several blocks of cat spinal cord and brain that had been hardened in 10% formalin for almost 3 years exhibited a marked refractoriness to perchloric acid extraction, considerable cytoplasmic and nucleolar basophilia remaining even after treatment for 1 hour at 37°C. This tissue also proved to be markedly resistant to ribonuclease digestion. It is apparent from Table I that liver cells and nerve cells from the same animal differ consistently with respect to PNA extractibility, although the same tissues from different species behaved similarly in this respect.

Extraction of DNA. Feulgen-stained control and experimental sections of dog liver were compared visually and photometrically after 18 hours and 93 hours of extraction in 10% perchloric acid at 4°C. No differences were observed visually in the intensity of nuclear staining. Photometric measurements, expressed as extinction coefficients, of a constant circular area 6.7 μ in diameter of liver cell nuclei in these sections confirmed the visual impression (Table II). Sections of spinal cord of cat incubated at 20°C and 37°C in perchloric acid for several time intervals along with control sections in water were similarly studied. No perceptible difference in the intensity of Feulgen coloration of glial nuclei was observed between control and experimental sections incubated for 16 hours at 20°C and for 15 minutes at 37°C. After 22 hours of extraction at 20°C and 20 minutes at 37°C, however, the experimental sections showed a perceptible reduction in Feulgen staining of nuclear chromatin. Photometry of a circular area 3.3 μ in diameter of astrocyte nuclei of the grey matter confirmed the visual impressions. Although little, if any, DNA has been extracted at the time PNA is removed, it is evident that DNA is rapidly removed after this point at higher temperatures.

Extraction of protein. Sections of cat spinal cord were extracted with 10% perchloric acid for 15 minutes at 37°C, at which time PNA extraction was complete. The intensity of coloration of such sections appeared the same as that of control sections incubated in water at 37°C in Millon preparations. Photometric measurements indicated that this extraction procedure did not remove significant quantities of protein from nerve cell cytoplasm in fixed deparaffinized sections of nervous tissue, the mean extinction of the control sections being .36 ± .04 as compared with .34 ± .02 for the experimental sections.

Effect on cell structure. No alterations could be detected in sections of spinal cord and liver as a result of perchloric acid extraction at 4°C, 20°C, and 37°C.

Effect on the extinction coefficient at 2537Å. The mean extinction coefficient of cat skeletal muscle at 2537Å was unaltered by the perchloric acid extraction procedure. Photographs taken at this wave length after this procedure were identical with those taken prior to treatment. The extraction procedure likewise did not alter the E2537Å of the ventral horn cells of the spinal cord that had been pretreated with ribonuclease to remove PNA. In nerve cells, cytoplasmic PNA accounted

TABLE III. Effect of Ribonuclease and Perchloric Acid Digestion on E_{2537}Å of Skeletal Muscle Fibers and Nerve Cells (Mean ± St. Dev. N=10).

Tissue	Before treatment	After ribonuclease	After perchloric acid
Muscle	.24 ± .01		.24 ± .04
Neurones	.45 ± .02	.25 ± .03	.26 ± .02

for only 44.5% of the total E2537Å (Table III).

Discussion. The perchloric acid procedure of Ogur and Rosen suffers from several limitations as a substitute for ribonuclease in the qualitative histochemistry of nucleic acid. Its basic shortcoming is its lack of specificity for PNA. When extraction is carried out at 4°C, DNA is not removed in significant quantities even after prolonged treatment. Such treatment, however, may depolymerize DNA without extracting it, as suggested by reduced stainability with methyl green(3). Several factors affect the extractability of PNA by perchloric acid and may require either a longer period of treatment at 4°C than the 18 hours suggested by Ogur and Rosen or higher temperatures for incubation. PNA may be different chemically or physicochemically in different organs of the same animals and these differences may be reflected in differences in perchloric acid extractability. This was found to be true for nerve and liver cells of the cat and dog. Species differences in PNA also may influence extractability. Cassel(4) indeed found that 30 hours of exposure to perchloric acid was required to remove all the PNA from a species of bacteria. Fixation can affect PNA extractability as was found to be the case for nervous tissue hardened for a long period of time in formalin. Inbedding in hot paraffin also can influence PNA extractability(5). In the application of this procedure to any particular fixed tissue, therefore, it may become necessary to determine the optimum conditions for extraction with perchloric acid that will give a complete removal of PNA with little or no loss of DNA. Where one is concerned only with cytoplasmic PNA, the problem is greatly simplified, inasmuch as DNA is not normally found outside nuclei.

Perchloric acid extraction would appear to lend itself admirably to quantitative ultraviolet microabsorption spectroscopy of PNA. As has been pointed out by Caspersson(14) and others, the extinction coefficients obtained in tissue sections near the nucleic acid absorption maximum of 2600Å are only partly attributable to specific absorption by purine and pyrimidine nucleotides. Non-specific light losses incurred by reflection, refraction, and scattering of light in an optically heterogeneous medium contribute to no small extent to the extinction coefficients obtained in tissue section in the short ultraviolet region. Tissue protein, while its absorption maximum is close to 2800Å, also exhibits considerable specific absorption at 2600Å. This value may be from one-half to two-thirds or more of the E_{2800}Å for protein. Since nucleic acid in tissue sections will almost always be found in conjunction with protein, the specific absorption of the latter may contribute appreciably to the total E_{2600}Å. Pollister and Ris(10) have suggested the use of a tissue "blank" for the evaluation of the above errors and have employed hot trichloracetic acid to provide such blanks. Pollister and Leuchtenberger (15) and Hamberger and Hyden(16) used crystalline ribonuclease for this purpose. That the perchloric acid extraction procedure is well designed for this purpose is supported by the following experimental evidence. This procedure did not remove appreciable amounts of tissue protein. It did not alter cell structure in fixed sections as seen with the phase contrast microscope or in sections stained with acid dyes. Finally, it did not influence the E_{2537}Å of muscle fibers, which contain no PNA, and nerve cells in which PNA had first been removed with crystalline ribonuclease.

Summary. (1) PNA was completely extracted from fixed sections of liver by 18 hours of treatment with 10% perchloric acid at 4°C. No significant quantity of DNA was removed by such treatment even after 93 hours of incubation. (2) The above treatment is inadequate to extract PNA completely from nerve cells. However, with formalin-fixed nerve tissue, 12 hours incubation in 10% perchloric acid at 20°C and 15 minutes at 37°C was found to effect complete extraction of PNA with no loss of DNA. (3) At higher temperatures of extraction, the margin of

safety between complete removal of PNA and incipient extraction of DNA was much smaller. (4) Prolonged hardening of nervous tissue in 10% formalin rendered PNA resistant to extraction by perchloric acid. (5) Perchloric acid extraction of fixed sections of nervous tissue removed insignificant quantities of nerve cell protein. (6) Cell structure was unaltered by this extraction procedure. (7) This extraction procedure did not alter the E_{2537}Å of two tissues without PNA, skeletal muscle and ribonuclease-treated nerve cells. It was suggested that this procedure could be employed to provide tissue "blanks" in ultraviolet microabsorption spectroscopy for PNA in tissue sections.

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Determination of Polyglucose in Blood and Urine.* (19308)

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Polyglucose is a water-soluble polymer of glucose prepared by chemical polymerization. Preparations with molecular weights ranging from 3000 to 160000 have been obtained. Animal experiments have indicated that solutions are non-toxic, and may have value for use as a blood substitute in treatment of shock.†

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† Material for the present studies was obtained by courtesy of the E. I. du Pont de Nemours and Co. through the Medical Research Committee of the National Research Council. This material was prepared by Dr. P. T. Mora of the Rayon Dept., Pioneering Research Laboratory, Dupont Experimental Station, Wilmington, Del. Sample P-24-14-PIa and sample P-24-52-2 were the samples used in the majority of the experiments described in this paper.

Composition of polyglucose used as basis for analytical calculations. Polyglucose, like starch and glycogen, is difficult to dry to an anhydrous condition. Air-dried preparations contain 10% or more of moisture. After drying 8 days at 80° at atmospheric pressure, a preparation reached constant weight. Its carbon content then was 42.37%, corresponding to $(C_6H_{10}O_5)_2 \cdot H_2O$ (theory, 42.1% carbon), rather than to $C_6H_{10}O_5$ (44.44% carbon). It is uncertain whether the half molecule of water per $C_6H_{10}O_5$ unit in the dried preparation was moisture that could not be driven off under the conditions used, or whether it was a part of the molecular structure. In starch and glycogen Dumazert(3) has found the same composition (C = 42.2 to 42.4%) when these carbohydrates were dried at 80° under less than 1 mm pressure. His

dried starch and glycogen showed zero water content by the chemical method of K. Fischer; consequently, it appeared that the H_2O of the residual $(C_6H_{10}O_5)_2 \cdot H_2O$ was part of the composition of these polysaccharides, which Dumazert accordingly expressed as $(C_{12}H_{22}O_{11})n$. The question is raised, whether it is preferable to base calculations of polyglucose on the anhydrous formula, $C_6H_{10}O_5$, or on $(C_6H_{10}O_5)_2 \cdot H_2O$. Because of uncertainty regarding the nature of the half molecule of water per $C_6H_{10}O_5$ unit in the apparently dried polyglucose, and because of the established use in analytical chemistry of the anhydrous formula in calculations of starch analyses, we have adhered to the conventional anhydrous formula $C_6H_{10}O_5$ in the present analyses.

Reactions used for determination of polyglucose.‡ Two reactions of polyglucose are applied. One is that with anthrone, which produces a green color, adaptable to photometry, with apparently all carbohydrates. The reagents and procedure are simple. The color intensity produced per unit of carbon has been found, by experiments with solutions standardized by carbon determination(10,11), to be the same whether the glucose is free or is combined in polyglucose. The other reaction is the increase in reducing sugar caused by acid hydrolysis. Polyglucose has some reducing power, but it is greatly increased by hydrolysis.

Reaction of anthrone with polyglucose and glucose. Dreywood(2) showed that when a solution of anthrone in concentrated sulfuric acid is added to one volume or less of aqueous carbohydrate solution a green color results, that the reaction is given by all types of carbohydrates, and by none other of the substances tested except glycerol and furfural. The reaction was placed on a quantitative basis for colorimetric analyses by Morris(7), and was used by Durham and coworkers(4) for the determination of blood sugar. Polyglucose reacts like the naturally occurring carbohydrates. The preparation of standard polyglucose solutions by weight is difficult because of the extremely hygroscopic nature of the substance. However, when the chromogenic power of polyglucose solutions was compared with that of glucose solutions containing the same weight of carbon per unit volume (10,11), it was found that the color intensities produced were exactly equal, and obeyed Beer's law. Hence, assuming $C_6H_{10}O_5$ as the composition of anhydrous polyglucose, 1.111 mg of glucose is equal in chromogenic power to 1 mg of polyglucose, and glucose solutions prepared on that basis can be used as standards for photometric determination of polyglucose. As found for other carbohydrates (1,2,4,7), polyglucose develops in 15 minutes a color with anthrone that remains constant for several hours at room temperature. However, the *conditions at the time of the color development* have an effect on the intensity of the color that has not been mentioned in previous publications. When 10 ml of anthrone solution in 95% sulfuric acid were added to 5 ml of aqueous polyglucose solution in a test tube cuvette of 17 mm bore, with shaking of the tube during the addition(4,7), duplicate photometric readings gave a standard deviation of 1.8% of the mean, which was several times the instrumental error. In order to obtain more rapid and uniform mixing, the anthrone solution was added to the carbohydrate in 50 ml Erlenmeyer flasks, with constant rotation, 75 seconds being taken for the addition. The result was an optical density only 60 to 70% as great as when the mixing occurred in the test tubes. The cause of the difference was not temperature, which was found to rise to 94-95° in both the tubes and the flasks. Various procedures of mixing were tried, including heating the cuvettes for 3 minutes in a water bath after the mixing. As constant an optical density per unit of polyglucose was obtained by the following procedure as by any more elaborate. To 5 ml of polyglucose solution in a test tube cuvette of 17 mm bore, 10 ml of anthrone solution were added from an automatic burette of that capacity with the cock fully opened so that addition was made during a constant

‡ The determination of polyglucose has also been studied at the Merck Institute for Therapeutic Research, Rahway, N. J., by R. H. Silber and H. J. Robinson. They have developed turbidimetric procedures for the determination. Their methods are as yet unpublished.

interval of 75 seconds. The anthrone solution was dropped directly onto the surface of the aqueous polyglucose solution, and the tube was shaken constantly during the addition and for a few seconds afterwards, until the disappearance of refraction differences in the solution indicated that mixture was complete. The tubes were then let stand at room temperature, and readings were made after 15 minutes. With amounts of polyglucose giving optical densities of 0.3 to 0.5 the standard deviation of replicates from the mean was ±1.8%. The dependence of the optical density on the conditions under which the two solutions are mixed makes it essential to run duplicate analyses, with the color developed in different cuvettes.

Reducing power of polyglucose before and after Hydrolysis. For measurement of reducing substance, the sugar method of Miller and Van Slyke(6) was used, in which the sugar acts on an excess of ferricyanide and the ferrocyanide formed is measured by titration with ceric sulfate. Polyglucose of average molecular weight about 90000 analyzed by this method, without preceding hydrolysis, showed per unit of carbon 3.4% of the reducing power shown by glucose. A preparation of average molecular weight about 8000 showed 9.7%. Per mg of polyglucose calculated as $C_6H_{10}O_5$, their reducing values are 3.7 and 10.6% of the reducing power of 1 mg of glucose. For determination of the rate of hydrolysis the polyglucose solution was mixed with an equal volume of 2 N hydrochloric acid and heated in a test tube immersed in a boiling water bath with the tube loosely covered to prevent loss of water by evaporation. Reducing power reached a plateau in 2 hours. Per mg of carbon the reducing power was then 92 ± 0.5% that of glucose, for both of the preparations mentioned in the preceding paragraph. With the higher molecular weight polyglucose preparation the increase in reducing power caused by hydrolysis was 92 − 3.4 = 88.6% of the theoretical increase that would have occurred if the reduction had been zero before hydrolysis and if all the combined glucose, estimated from the carbon content, had been set free by the hydrolysis. With the lower molecular weight preparation the increase was 92 − 9.7 = 82.3% of theoretical. Failure to obtain theoretical yields of glucose by acid hydrolysis has been noted with starch and glycogen. In a recent careful study Dumazert(3) has found that the maximal reducing power obtained from both of these carbohydrates by hydrolysis was 95% of that calculated from the carbon content on the assumption of complete conversion to glucose. The increase in reducing power caused by hydrolysis serves to measure polyglucose in the presence of pre-formed glucose, and is the procedure used at present for blood analysis.

Photometric determination of polyglucose in glucose-free urine with anthrone. In the absence of glycosuria the anthrone method is the one of choice for urine. The non-carbohydrate reducing substances of urine, which in their reaction with the reagents used for reducing sugars may be equivalent to as much as 2 grams of sugar per liter of urine(8,9), do not give an appreciable color with anthrone.§

Reagents. Anthrone solution. 1 g of anthrone is dissolved in 95% sulfuric acid, prepared by mixing 475 ml of sulfuric acid of specific gravity 1.84 with 25 ml of water. The solution is stored in a refrigerator. It has remained satisfactory for several weeks. The anthrone used was obtained from the Montclair Research Corp., Montclair, N. J. The blank obtained with different batches of sulfuric acid varied considerably. A bottle of Eimer and Amend's "C.P. Tested Purity Reagent" was satisfactory. *Stock glucose solution.* 111.1 mg of glucose are dissolved in water and made up to 100 ml. In chromogenic power with anthrone this is equivalent to a solution of 1 mg of anhydrous polyglucose, calculated as $C_6H_{10}O_5$, per ml. *Determination of standard optical density or transmission curve.* Portions of 1, 2, 3, and 4 ml of the stock glucose solution are diluted

§ Methods for determining polyglucose in glucose-containing urine have not been tested. Presumably the anthrone procedure could be used for total carbohydrates, with subtraction of 0.9 of the glucose to calculate polyglucose. However, because of non-glucose reducing substances of urine, the correction would have to be made by determining the glucose by fermentation(8,9).

each to 100 ml. Of these solutions triplicate portions of 5 ml, containing glucose chromogenically equivalent to 0.05, 0.10, 0.15, and 0.20 mg of polyglucose, respectively, are pipetted into cuvettes. To each solution in its cuvette 10 ml of the anthrone solution are added slowly with shaking. A blank solution is prepared by adding 10 ml of anthrone solution to 5 ml of water in another cuvette. After the mixtures have stood 15 minutes or longer, readings in a photometer are taken of transmission or optical density with light of 630 millimicrons wave length, the blank being used to set the zero point. For the standard curve, optical densities (optical density = 2 – log of percentage transmission) or the logarithms of the transmissions are plotted against the equivalents of mg of polyglucose in the cuvettes, 1.111 mg of glucose being equivalent to 1 mg of polyglucose. If the scale of the photometer reads directly in optical densities, it is convenient, instead of plotting the linear curve, to use the readings to determine the K value of the equation: (1) Mg polyglucose in cuvette = KD where D is the optical density.

In the present work, the photometer used was a Coleman Junior, with a density as well as a transmission scale. The cuvettes were Pyrex test tubes, 19 by 150 mm, tested for uniformity of transmission. With this equipment the K value of Equation 1 was 0.239, with a standard deviation of ±0.004, or 1.8%.

Analysis of urine. Dilution. Urine is so diluted that 5 ml of the diluted solution contain about 0.1 mg of polyglucose. If x = mg of polyglucose expected per ml of urine, one volume of urine is diluted to the nearest convenient approximation of 50 x volumes. *Color development and reading.* Duplicate 5 ml portions of the diluted urine are treated with anthrone and the photometer readings are taken as described for the standard curve. *Calculation.* The mg of polyglucose in the cuvette are estimated by interpolation on the standard transmission curve, or from optical density by Equation 1 and the concentration of polyglucose in the urine is calculated as: (2) Mg polyglucose per ml urine = mg in cuvette x 0.2 n where n is the number of times the urine was diluted before the 5 ml sample was taken.

Determination of polyglucose in blood or plasma. Bloom and Willcox(1) have used anthrone for blood dextran, with preliminary quantitative separation of the dextran from glucose by the procedure commonly used for glycogen in tissues, the material being digested with strong KOH solution, and the polysaccharide in the digest precipitated by alcohol in approximately 60% concentration. This procedure was not found applicable to these particular samples of polyglucose, because precipitation was incomplete. For accurate results the polyglucose has been determined by measurement of the reducing sugar before and after acid hydrolysis. The reducing sugar has been determined by the ferricyanide reduction method of Miller and Van Slyke(6). The ferrocyanide formed by reduction is titrated with standard ceric sulfate solution, of which 1 ml is equivalent to 0.1 mg of glucose. The method has a precision of 1%. The range of glucose covered is wide, going up to the equivalent of 8 mg of glucose per ml of blood, which is of advantage for the great range of polyglucose concentrations that is encountered after infusions. The glucose equivalent of the ferricyanide reagent is not affected by the NaCl present in the hydrolyzed samples. Presumably any other reduction method with similar advantages would serve. For rapid approximate analyses the anthrone method can be applied to the Folin-Wu tungstic acid filtrate, the total carbohydrate thus determined being corrected by subtracting 1.0 mg per ml of blood for the usual amount of glucose plus polysaccharide present in normal blood. The correction can be made more exact by determining the blood glucose, but this procedure, involving two different types of analysis and control of their reagents, offers no advantage in convenience or speed over the hydrolysis method, and is likely to be less accurate. Polyglucose penetrates the cells to a negligible extent. The plasma polyglucose can either be determined directly, or can be estimated by multiplying the blood concentration by 100/(100 – hematocrit). For precipitation of blood proteins to obtain a filtrate for polyglucose determination, we have used the Folin-Wu tungstic acid method.

Protein precipitates formed with zinc or cadmium hydroxides carry down considerable amounts of polyglucose.

Reagents. Redistilled water. Ordinary distilled water may contain contaminants that reduce significant amounts of the dilute standard cerate solution on standing. Hence, redistilled water is used to prepare the standard cerate solutions. Distilled water is treated with 5 ml of concentrated sulfuric acid and 5 g of potassium dichromate per liter, and is redistilled. *Ten per cent sodium tungstate.* Ten grams of $Na_2WO_4 \cdot 2H_2O$ per 100 ml. Deviation of the solution from neutrality is tested by adding a drop of phenolphthalein to 10 ml of the solution and titrating with either 0.1 N H_2SO_4 or NaOH to neutrality. If the solution is alkaline and requires more than 0.4 ml of 0.1 N acid, enough 1 N H_2SO_4 is added to the stock solution to render it neutral to phenolphthalein. If the solution is acid, it is similarly neutralized with 1 N NaOH. *1/12 N sulfuric acid. Tungstic acid solution*(9). One volume of the tungstate is mixed with 8 volumes of 1/12 N sulfuric acid. The solution slowly loses strength by precipitation of the tungstic acid, and should be prepared fresh every two weeks. *2 N hydrochloric acid. 1 N sodium hydroxide.* This is adjusted by titration against the 2 N HCl so that 2 volumes of the alkali are equivalent to 1 volume of the acid. *Alkaline ferricyanide solution*(6). 5 g of recrystallized, reagent grade, $K_3Fe(CN)_6$ and 10.6 g of anhydrous Na_2CO_3 are dissolved in water and made up to 1 liter. The solution must be free of ferrocyanide. The presence of the latter will be revealed by a high blank titration. Ferrocyanide can be removed by the recrystallization process of Folin(4a). *18 normal sulfuric acid (approximate)*(6). Add 465 ml of concentrated sulfuric acid (sp. g. 1.84) to 535 ml of water. Reagent grade sulfuric acid must be used, and must be first tested for reducing material as follows: To 20 ml of the acid add 60 ml of water and 0.05 ml of approximately 0.1 N $KMnO_4$ solution. The pink color must last for at least 5 minutes. *Indicator solution*(5). Dissolve 6.95 g (0.025 moles) of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in water and make up to 1 liter. Into the solution stir 14.85 g (0.075 mole) of ortho-phenanthroline monohydrate ($C_{12}H_8N_2 \cdot H_2O$) until all is dissolved. The indicator, either in solid form or prepared in solution as above, can be obtained from the G. Frederick Smith Co., Columbus, Ohio. In the sugar titration the color change at the end point is sharp, from golden brown to light yellow. On standing, the golden brown slowly returns. The end point is, however, stable for at least a minute. For use in the titrations, the above stock solution is diluted 5-fold with water. *Stock 0.1377 solution of ceric sulfate*(6). Approximately 110 g of anhydrous ceric sulfate (obtainable from the G. Frederick Smith Co.) is placed in an 800 ml beaker, and 35 ml of concentrated H_2SO_4 and 35 ml of water are added. The mixture is stirred and heated, and small amounts of water are added until practically all of the ceric sulfate is dissolved. The solution is filtered, cooled, and the clear filtrate diluted to 1 liter. The solution is standardized by titration against an 0.1 N solution of ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$), which is prepared by adding 39.214 g of the salt (analytical reagent grade, for use as analytical standard) to 200 to 300 ml of water, adding 25 ml of the 18 N H_2SO_4, and dissolving with stirring. The solution is then diluted to 1 liter. For the standardization of the ceric sulfate solution, 15 ml of the latter are pipetted into a 150 ml beaker, diluted with 50 ml of water and 3 ml of 18 N H_2SO_4, and titrated with the ferrous ammonium sulfate solution until the yellow color almost disappears. Then 1 or 2 drops of the diluted indicator solution are added and the titration is continued until the color changes from light yellow to golden brown. The normality of the Ce^{IV} solution is calculated as (ml ferrous salt solution)/(ml Ce^{IV} solution) $\times$ 0.1. The ceric sulfate should be more than 0.15 N. From it an exactly 0.1377 N solution is made by dilution with distilled water. *E.g.*, if the normality is 0.1560, then (0.1377/0.1560) $\times$ 1000 = 883 ml are diluted to 1 liter to make the 0.1377 N solution. The stock solution stored in a dark bottle will keep for a year. It should be kept at room temperature, not in the ice box, as some of the ceric salt may crystallize out in the cold.

TABLE I. Volume of 1:10 Blood or Plasma Filtrate to Take for Analysis.

mg polyglucose/ml of blood or plasma	ml of filtrate to use for titration samples (V of equations 4 & 5)	ml of filtrate to dilute to 25 ml for anthrone method (W of equation 6)
<1	2	5
1 to 3	1	2
3 to 8	.5	1
>8	.2	.3

0.002754 N standard ceric sulfate for blood sugar titrations(6). Exactly 2.000 ml of the stock solution are pipetted into a 100 ml volumetric flask. Five ml of the 18 N H_2SO_4 solution are added, and the solution is made up to 100 ml with the *redistilled water*. If the dilution is made with ordinary distilled water, the strength of the ceric sulfate may decrease measurably within 2 or 3 hours. If prepared with water redistilled as directed, the strength does not change significantly for 6 hours at room temperature in diffuse daylight, and will not change in a week in a refrigerator.

Analysis of blood or plasma. Precipitation of proteins. One volume of blood or plasma is run with stirring into 9 volumes of tungstic acid solution. Filtration is through either paper or cotton, either of which should be prepared by previous washing and drying. *Measurement of filtrate samples.* Aliquots of tungstate filtrate are measured to contain preferably not over 0.5 mg of polyglucose. The titration method can handle samples containing up to 0.8 mg of total glucose, but it is convenient to keep the total of polyglucose plus blood glucose in the sample below 0.5 mg, so that the volume of cerate solution required will be under 5 ml. From the expected concentration of polyglucose in the blood or plasma, the volume of desirable aliquot of the filtrate to take as sample may be estimated from the second column Table I.

Preparation of samples with and without hydrolysis. For analyses in duplicate, measure into each of four 40 ml heavy-walled test tubes the volume of filtrate estimated by Table I, and add from a burette enough water to bring the volume up to 3 ml. Two of the tubes are used for duplicate determination of reducing sugar without hydrolysis, the other 2 for determination after hydrolysis. To each of the 2 tubes for hydrolysis add 3.00 ml of 2 N hydrochloric acid. Immerse the tubes, loosely covered, in boiling water for 2 hours. Then cool, add 3 ml of water, and neutralize by adding 6.00 ml of the 1 N sodium hydroxide. To the other 2 tubes, for analysis without hydrolysis, add 12 ml portions of redistilled water. *Reduction.* To each of all 4 tubes add 2 ml of the alkaline ferricyanide solution. Mix by gently whirling the tubes. Place all the tubes in a rack or wire basket which can be immersed in a boiling water bath deeply enough to immerse the tubes at least as far as the level of the surfaces of the solutions. The tubes must be so arranged that they do not touch each other. If a wire basket is used, it should have a raised floor. The tubes are immersed in actively boiling water for 15 minutes, then cooled at once by immersion in running water for 3 minutes. *Titration of the ferrocyanide formed.* The titration should be performed within an hour after the reduction. Just before each solution is titrated, approximately 1 ml of the 18 N H_2SO_4 is added, followed by one drop of the diluted indicator solution. The solution is then titrated with the 0.002754 N ceric sulfate. The titration must be performed in a white light against a white background. A rapid stream of the ceric sulfate may be run in until the color begins to change. *Blanks.* Two blanks are determined, one for the analysis without hydrolysis and one for the analysis with hydrolysis. For each the blood filtrate is replaced by water, and for the blank for the hydrolyzed filtrate the amounts of HCl and NaOH used for the latter are employed. The usual blank found for the unhydrolyzed solution has been 0.10 ml of the cerate solution, for the hydrolyzed solution 0.14 ml of the cerate. The blanks, once determined, serve for analyses until the reagents or filter papers, used for filtering the blood filtrates, are changed.

Calculation. The titrations are made with cerate solution of which 1 ml is equivalent to 0.1 mg of glucose. H = ml of cerate, minus the corresponding blank, to titrate the hydrolyzed sample. U = ml of cerate, minus the corresponding blank, to titrate the unhydro-

lyzed sample. f = % of theoretical increase in reducing power caused by hydrolysis of the polyglucose used. (See "Reducing power of polyglucose before and after hydrolysis.") V = volume in ml of 1:10 filtrate used as sample. The general equation is: (3) Polyglucose in sample $= 0.9\Delta \times \frac{100}{f}$ where Δ = increase in reducing sugar, calculated as glucose. When the Miller-Van Slyke titration is used as above described, $\Delta = 0.1(H-U)$, and the ml of blood represented in the sample = 0.1 V. Hence: (4) Mg polyglucose per ml blood or plasma $= \frac{H-U}{V} \times \frac{90}{f}$ For the higher molecular polyglucose tested, similar to that which will probably be used for infusions, f was found to be 88.6. For this value of f the calculation becomes: (5) Mg polyglucose per ml blood or plasma $= 1.016 \frac{H-U}{V}$.

To correct for the slight amount of naturally occurring polysaccharide present in the blood, a determination of the increase in reducing power caused by hydrolysis can be made on a sample of blood drawn before the polyglucose infusion, and the amount found can be subtracted from the polyglucose calculated by Equation 4 or 5. Since the correction is not usually over 0.10 or 0.12 mg per ml of blood (1), it can be neglected, except for accurate estimations of small concentrations of polyglucose.

Approximate determination of polyglucose in blood or plasma with anthrone. Reagents. Anthrone solution, described for urine analyses. *Tungstic acid solution*, described above.

Analysis of blood or plasma. Precipitation of proteins with tungstic acid as described for the reduction procedure. *Dilution of the tungstic acid filtrate.* The volume of filtrate, W, indicated in the last column of Table I is diluted to 25 ml with water. *Determination of total polyglucose plus glucose in filtrate.* Five ml portions of the diluted filtrate are treated with 10 ml portions of anthrone solution, and the optical density read as described for urine. *Calculation.* The "mg of polyglucose in the cuvette" are calculated as described for urine analysis. (6) Mg polyglucose per ml blood $= (\frac{50}{W} \times$ "mg polyglucose in cuvette") — 0.9 G, where W is the volume of tungstate filtrate diluted to 25 ml before the 5 ml portion was taken for analysis, and G represents preformed blood carbohydrate in mg per ml. Ordinarily G can be taken as 1 mg per ml of blood or plasma.

Precaution in use of anthrone method. Anthrone develops color with minute amounts of all carbohydrates. There is enough cellulose in dust and lints to affect analyses. All glassware must be kept clean and free from dust, and the reagents must be protected from dust.

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Effect of Hypophysectomy on Blood Regeneration in the Adult Female Rat.* (19309)

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It is well known that hypophysectomy induces an anemia in a variety of laboratory animals (dog, rabbit, cat), although most of the recent literature on the pituitary-blood relationship is confined to the rat. In addition, most investigators have found that an anemia accompanies panhypopituitarism in humans. Several investigators have studied hemopoietic activity in the hypophysectomized rat following subjection to anoxia, believed by some to be the fundamental erythrocytogenic stimulus. Stewart, Greep, and Meyer(1) found that hypophysectomized rats exposed to lowered barometric pressures (422 mm Hg) did not exhibit bone marrow hyperplasia, reticulocytosis, and an increased red cell count as did normal animals subjected to the same decrease in oxygen tension. In a more recent report, Feigin and Gordon(2) confirmed the finding that hypophysectomized rats do not respond to a barometric pressure of 422 mm Hg, but found that they would respond if the pressure was reduced to 320 mm Hg. Under these conditions, a blood and bone marrow reaction was evoked in hypophysectomized rats which was equivalent to that of normal rats subjected to such a pressure. The report concluded that the pituitary is not essential for an erythropoietic response to severe anoxia and that the negative response to moderate anoxia exhibited by the hypophysectomized animal was due probably to its lowered basal metabolic rate and lower oxygen requirements. Bleeding is a form of erythropoietic stimulus akin to the anoxia achieved by exposure to lowered barometric pressures. The bled rat has been used previously as a sensitive test animal for the study of hemopoietic activity following hypophysectomy. Querido and Overbeek(3) and Finkelstein and coworkers(4) noted strong reticulocytosis and recovery from hemorrhagic anemia in hypophysectomized-bled rats.

The present investigation is a highly controlled, quantitative study of blood regeneration in the hypophysectomized rat following induced hemorrhage. Its purpose is to verify and extend previous work on the subject.

Materials and methods. Female rats of the Wistar strain, 2 to 3 months of age and weighing 140-253 g at the start of the experiment, were used in this study. All rats were fed Purina Laboratory Chow, supplemented by lettuce given weekly, and tap water *ad libitum*. Hypophysectomy was performed parapharyngeally under ether anesthesia. Completeness of pituitary removal was checked at autopsy by careful examination of the pituitary capsule under a dissecting microscope. The adrenals were removed and wet weights determined. Marked atrophy of these glands in the operated rats served as additional evidence for complete removal of the pituitary. The operation was complete in all hypophysectomized rats included in this work. Hemorrhagic anemia was produced by removal of blood by means of cardiac puncture. The heart was entered with a sharpened 23-gauge hypodermic needle. Some of the rats were rendered anemic by bleeding from the tail which was immersed in a warm solution of 1.3% sodium oxalate. The heart and tail methods were equivalent for production of hemorrhagic anemia. In order to produce a similar degree of anemia from one animal to the next, the volume of blood withdrawn from each animal was determined upon the basis of the weight of the animal and the hematocrit on the day of bleeding. The number of cubic centimeters of blood to be withdrawn was calculated ac-

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cording to the following formula: $cc = .02 \times \text{body wt in g} \times \frac{\text{hematocrit}}{45}$. Preliminary experimentation led to formulation of this equation. It was found that bleeding 2% of body weight induced a definite hemorrhagic anemia. The hematocrit was included in the equation to compensate for the fewer red cells in the hypophysectomized rat. The 45 in the denominator was the mean hematocrit of 38 normal rats. Peripheral blood from the tail was used throughout the study for purposes of red cell count, hemoglobin, and hematocrit determinations. There was no "milking" of the tail whatsoever. The tail was clipped with scissors and the first drop of blood discarded. Four to 5 drops were placed in a small vial containing a small amount of powdered heparin. Every rat was weighed on the day of hypophysectomy and on each day that a blood sample was taken. Erythrocyte counts were made in duplicate with standard U. S. certified pipettes and the Levy-Hausser counting chamber with the improved Neubauer double ruling. Hemoglobin concentrations were determined directly in grams of hemoglobin per 100 ml of blood using a Klett-Summerson photoelectric colorimeter with a green filter having an approximate spectral range of 500-570 millimicrons. Hematocrit determinations were made with Van Allen tubes which were centrifuged for one hour at 2000 RPM. Four types of control animals were used in this study. The first group consisted of unoperated-unbled (*i.e.*, normal) rats to determine normal variation in the blood picture. The second group consisted of hypophysectomized-unbled rats to show the blood changes following hypophysectomy. The third group consisted of unoperated-bled rats to indicate blood regeneration in the normal animal following induced hemorrhagic anemia. The final control group consisted of sham-operated-bled rats to show whether the surgical procedure involved in hypophysectomy influenced blood regeneration following bleeding. The experimental or hypophysectomized-bled rats consisted of two main groups. A hemorrhagic anemia was induced 10 days post-hypophysectomy in one group and 20 days post-hypophysectomy in the other group. There were 3 small supplementary groups, consisting of 2 or 3 rats each. These small groups were bled at 5, 30, and 40 days post-hypophysectomy. Blood samples were taken at 10-day intervals in all rats. In the bled rats, blood samples were taken at 10-day intervals to the day of bleeding, at one day post bleeding, and at 10-day intervals thereafter. Initial blood samples were taken on all animals before subjection to any procedures whatever.

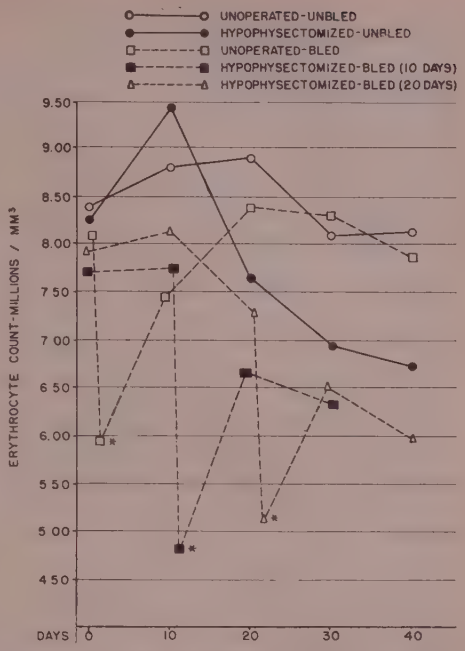

FIG. 1. Average erythrocyte counts of all groups throughout the course of the experiment. * Bled one day previous.

Results. Fig. 1, 2, and 3 give the results in graphic form.

Hypophysectomized-unbled—5 rats. The erythrocyte count, hemoglobin, and hematocrit values decreased to 81.6%, 80.5%, and 77.8% of normal, respectively, over the 40-day period following hypophysectomy.

Unoperated-bled—8 rats. The figures show that bleeding caused a 27.1% drop in erythrocyte count, a 26.2% drop in hemoglobin, and a 23.4% drop in hematocrit, *i.e.*, a drop to values 72.9%, 73.8%, and 76.6% of normal for erythrocyte count, hemoglobin, and hematocrit, respectively. Ten days post-

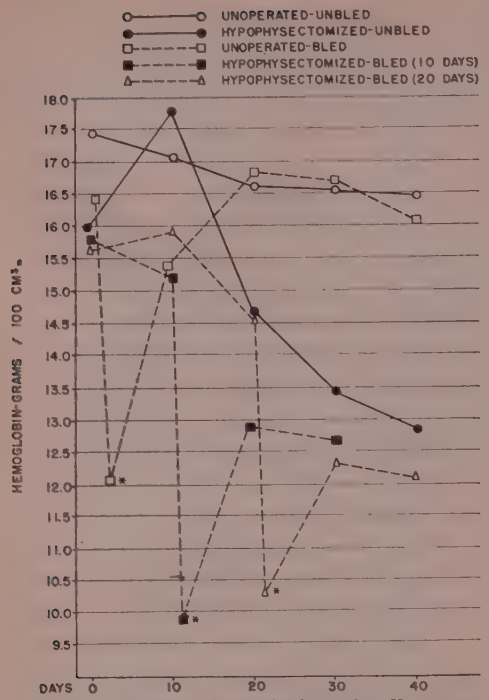

FIG. 2. Average hemoglobin of all groups throughout the course of the experiment. * Bled one day previous.

bleeding, the erythrocyte count, hemoglobin, and hematocrit had increased to values 91.9%, 93.9%, and 95.7% of normal, respectively. This is practically a complete recovery. In addition to the foregoing, 6 sham operated rats recovered completely (within 10 days) from the anemia induced by bleeding.

Hypophysectomized-bled (10 days after the operation)—8 rats. Bleeding in this group caused a decrease in erythrocyte count to a value of 61.8%, a decrease in hemoglobin to 64.7%, and a decrease in hematocrit to 65.1% of the pre-bleeding levels on the day after bleeding. On the 10th day after bleeding, the values for erythrocyte count, hemoglobin, and hematocrit had increased to values 85.8%, 84.3%, and 83.7% of pre-bleeding levels, respectively. This is incomplete regeneration. Since no further increases were noted at 20 and 30 days post-bleeding, it is apparent that maximum regeneration was reached 10 days following bleeding.

Hypophysectomized-bled (20 days after the operation)—6 rats. These rats showed a drop in erythrocyte count to 70.1%, a drop in hemoglobin to 71.0%, and a drop in hematocrit to 69.2% of pre-bleeding values on the day following bleeding. Ten days after bleeding, the values had increased to 89.1%, 84.8%, and 84.6% of pre-bleeding levels for the erythrocyte count, hemoglobin, and hematocrit, respectively. This is incomplete regeneration or incomplete recovery from bleeding. No further increase in blood values was seen at 20 days after bleeding; maximum regeneration was reached at 10 days post-bleeding. The pattern of incomplete recovery from bleeding in rats bled 20 days after the operation is similar to the incomplete recovery in rats bled 10 days after the operation. Both groups are in marked contrast to the unoperated-bled and the sham operated-bled rats which recovered completely from induced hemorrhagic anemia within 10 days. All of the foregoing data were treated statistically and the results are significant. In addition, small supplementary groups of rats (2 or 3 rats per group) were bled at 5, 30, and 40

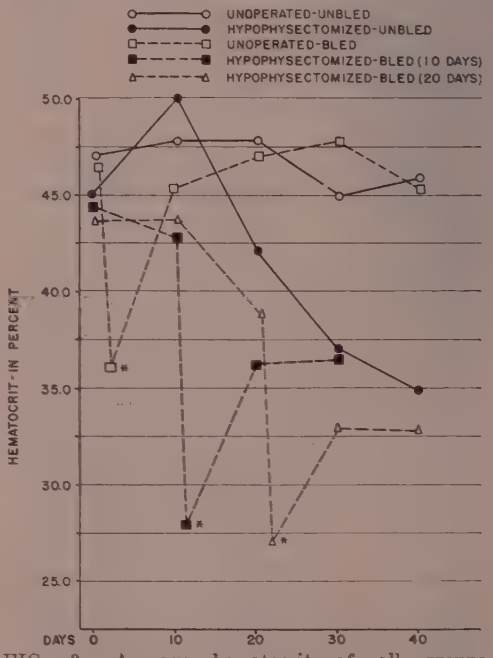

FIG. 3. Average hematocrit of all groups throughout the course of the experiment. * Bled one day previous.

days post-hypophysectomy. These animals showed the same general picture as seen in the rats bled 10 and 20 days post-hypophysectomy. Thus, while pre-bleeding values varied according to the number of days post-hypophysectomy, regeneration to the maximum level occurred in each instance within 10 days after the bleeding.

Discussion and conclusions. Within 10 days, the normal or sham operated rat recovers completely from the hemorrhagic anemia induced by bleeding to the extent of approximately 2% of body weight. The hypophysectomized rat can partially regenerate red cells lost by bleeding to the same extent. Regeneration is essentially completed during the first 10 days after bleeding. It has been shown by Crafts(5) that in the hypophysectomized rat the anemia becomes most severe at 40 days after operation and remains relatively constant thereafter. This has been called the plateau level. The present investigation has demonstrated that bleeding the hypophysectomized rat prior to the 40th day post-operative produces, as would be expected, a superimposed hemorrhagic anemia. Interestingly enough, the maximum blood level to which the hypophysectomized rat may regenerate by the 10th day after bleeding is similar to this plateau level of unbled-hypophysectomized rats. This recovery to the plateau level in hypophysectomized-bled rats occurs independent of the number of days after operation at which hemorrhage occurs.

It is likely that the incomplete response to anoxia exhibited by the hypophysectomized rat is not due directly to deprivation of any specific hemopoietic factor of the anterior pituitary. Hypophyseal control of the endocrines which regulate metabolism offers a more probable explanation. Similarly, the anemia of hypopituitarism is likely due to general metabolic upsets following hypophysectomy, since this anemia can be prevented completely by high protein diet-thyroxine-androgen therapy(5). Since incomplete response to anoxia exhibited by the hypophysectomized rat is due probably to alterations in metabolism, further investigation utilizing hypophysectomized rats treated with the high protein diet-thyroxine-androgen therapy of Crafts is in order. If such treated rats exhibit the normal polycythemic response at an atmospheric pressure of 422 mm Hg and show complete regeneration following bleeding, the metabolic explanation of post-hypophysectomy anemia will be further enhanced.

Summary. Intact normal rats and sham operated rats recovered completely from hemorrhagic anemia within 10 days. Hypophysectomized rats bled at various intervals after the operation exhibited partial regeneration of blood elements on the 10th day after bleeding. Maximal recovery was reached at that time with no further regeneration occurring subsequently. This maximum is identical with the plateau level which the hypophysectomized-unbled rat attains approximately 40 days after hypophysectomy. It is likely that the incomplete regeneration is due to the altered metabolism following hypophysectomy.

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Toxicity of Tritium Oxide to Mice. (19310)

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Tritium (H^3) is, in common with carbon-14, a radioisotope that may be used to label a large variety of physiologically important compounds. The soft nature of its β ray (mean energy about 5.7 kev) suggests interesting possibilities in the fields of radiobiology and therapeutics that might be achieved by selective localization in living structures. The biological effectiveness of this soft β ray is unknown, but the structures and functions sensitive to density of ionization appear to be predominant in determining radiation toxicity to the higher animals(1), so that direct experimental evidence regarding the biological effectiveness of tritium is important. Because of the low solubility of tritium or hydrogen gas in body fluids, it may be assumed that tritium water would be a considerably greater health hazard. These experiments were therefore done to establish the lethal dose of tritium water when injected intraperitoneally in physiological saline solution into CF No. 1 mice. Circumstances of the experimental conditions do not enable us to make rigid comparison of relative biological effectiveness as against better studied radiations, but do indicate clearly that no serious or unexpected correction factor needs to be introduced in calculating short-term tritium water toxicity on physical grounds.

Methods. Young female CF No. 1 mice, whose radiotoxic responses have been widely established in this laboratory, were injected intraperitoneally with 0.85% NaCl solution containing various amounts of tritium water diluted with sterile distilled water. Injection volumes ranged between 0.2 and 0.5 ml, and care was taken that none of the injection solutions escaped after removal of the needle. The mice were given water and Derwood checkers *ad libitum* and the intakes were measured daily. In a pilot experiment dosages of H_2^3O between 3.4 and 226 mc were given to mice weighing between 15 and 18 g; a subsequent experiment utilized mice weighing 20 $\pm$ 2.5 g, given dosages between 10 and 30 mc, bracketing the acute lethal range. The mortality results are shown in Table I.

Results. The tabulated results indicate a 30-day LD_{50} of about 1 mc per g body weight. Autopsy findings and hematologic observations indicate that deaths at and above this level were due to acute radiation disease.

Dosage calculations in physical terms would require serial analyses of body water specific activity and of uptake of tritium by organic compounds, but can be approximated by measurements of food and water intake. The

TABLE I. Mortality in CF #1 Mice Following Injection of H_2^3O.

Total dose, mc	No. of mice	Mice dying in 30 days		Median time to death
		No.	%	
	Exp. T 1: mean wt of mice 16.5 g			
226	2	2	100	90 hr
113	2	2	100	96
34	4	4	100	7.5 days
11.3	4	0	0	
3.4	4	1	25	(at 13 days; probably not radiation death)
	Exp. T 2: mean wt of mice 20 g			
34	4	4	100	12 days
28.3	6	6	100	12
22.6	5	3	60	16
17	6	1	17	12
11.3	6	1	17	16
Controls	7	0	0	

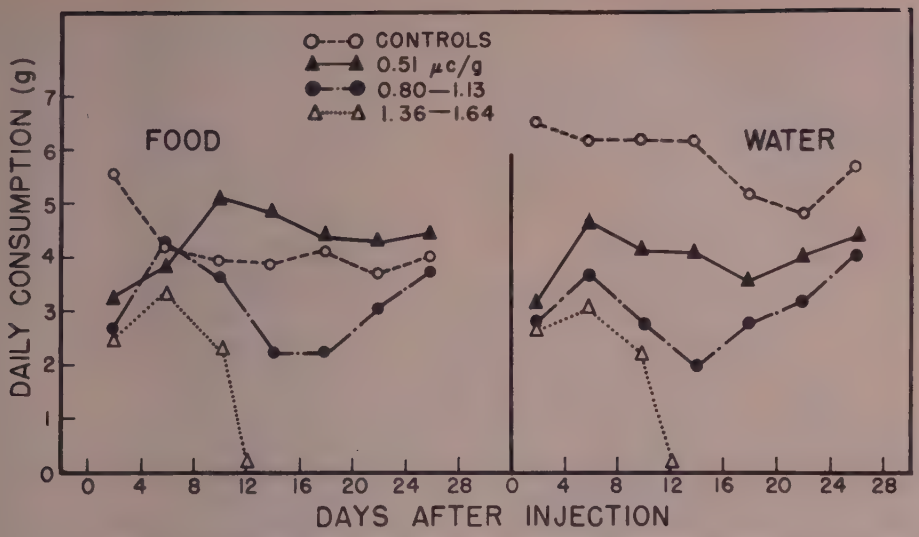

FIG. 1. Mean daily consumption of water and food by control and experimental mice divided into dosage groups as shown in the insert.

calculated total water intake (including metabolic water from food) in the mice receiving 20 and 15 mc in the second experiment indicated a rather constant water turnover for the first 4 days of 25% of the body weight per day, and for the second 4-day period this rose to about 42% per day. In Fig. 1 is shown the mean daily consumption of water and food in the various groups. The data indicate that treatment reduces the water intake more than the food intake, and that in the median lethal range food and water consumption are reduced most strikingly at the end of the second week. Assuming the body to be 75% water and that tritium oxide is excreted at the same rate as water, and disregarding the retarding effect of metabolic turnover of hydrogen and tritium, we have calculated the integrated total-body dosage over various periods of time at the several injection levels. These calculations appear in Table II. The 30-day LD_{50} for 250 kv X ray in these mice is about 575 r. Although no figures are available for estimating the lethal dose of X or γ rays administered with a time pattern such as this, it may be concluded that no remarkable factor of biological effectiveness exists for tritium β rays in relation to these better known radiations.

It is worth while remarking that dosages of tritium water in the lethal range tend to perpetuate themselves in the body due to the decreased food and water intake characteristic of the acute radiation response. This is illustrated by the relative rates of decrease in calculated dosage appearing in Table II. It should also be recognized that the relatively slower water turnover of the human being would result in a greater retention of the isotope and an increased toxicity, perhaps by a factor of 3 or 4, unless appropriate therapeutic measures were used.

Summary. The 30-day median lethal dose

TABLE II. Estimated Total-Body Dosage from Single Inj. of H^3_2O in CF #1 Female Mice.

Dosages, mc	mc/g	Initial rep/day	Dosages in rep 1-4 days	5-8 days	9-12 days	Total 1-12 days
11.3	.51	177	360	62	7	429
17	.80	264	605	155	18	778
22.6	1.13	353	812	206	24	1042
28.3	1.36	442	904	254	63	1221
34	1.64	530	1199	326	107	1632

of H_2^3O for CF No. 1 female mice is about 1 mc per g body weight when given in a single injection. The estimated radiation dosage is about 800 rep in the first 3 days and about 1000 rep in the first 12 days after injection. It is concluded that no unexpected factor of biological effectiveness exists for tritium β rays in relation to X or γ rays.

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Cation Exchange Properties of Alginic Acid. (19311)

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The ability of cation exchange resins to withdraw sodium from the body(1) suggested the investigation of cation adsorbing properties of various naturally occurring substances. This report summarizes the results of *in vitro* experiments conducted to determine the cation adsorbing capacity and ion selectivity of alginic acid. For comparison, similar studies were made with a carboxylic type cation exchange resin Natrinil.*

Alginic acid is obtained from the giant kelp (*Macrocystis pyrifera*). It is a polymeric anhydro-β-D-mannuronic acid chemically related to cellulose and pectic acid. Alginic acid possesses one carboxy group for each anhydromannuronic acid residue, and this free carboxy group is responsible for its ability to form water soluble gels with alkali metal ions. Alginic acid has a theoretical cation combining capacity of 5.7 meq/g.

The sodium and potassium salts of alginic acid are readily soluble in water. For this reason, separation of uncombined cations from the soluble alginate salts cannot be made by the use of the ordinary filtration or centrifuging technics generally employed with cation exchange resins whose alkali metal salts are insoluble. Dialysis of the mixture through a suitable semipermeable membrane such as cellophane provides a satisfactory method of effecting the separation of the diffusible uncombined cations from the adsorbed cations.

* Natrinil is the trade mark of the National Drug Co. brand carboxylic type cationic exchange resin. In all experiments reported here, the acid form of this resin was employed.

Determinations of sodium, potassium and calcium were made using a Perkin-Elmer Model 52-C Flame Photometer. In determining calcium the characteristic calcium oxide band spectrum was employed. In each procedure described, suitable control experiments were conducted to establish the validity of the method under the conditions utilized.

1. *Adsorption of Na^+ from sodium chloride solutions.* The weighed sample of alginic acid was allowed to equilibrate with a sodium chloride solution by shaking at room temperature for 3 hours. The mixture was then dialysed against 3 successive portions of 1 liter of water for a total period of about 40 hours. Control experiments indicated that these dialysis conditions permitted practically quantitative diffusion of the uncombined cations with negligible diffusion of alkali metal alginates. Sodium determinations were made on the supernatant liquid obtained by centrifuging the dialysis bag contents. Curves 3 and 4 of Fig. 1 show the amount of Na^+ adsorbed by 1 g alginic acid or 1 g Natrinil from solutions containing varying amounts of Na^+. The total volume of solution in each instance was 40 ml. Both alginic acid and Natrinil were poor sodium adsorbents in unbuffered sodium chloride solutions, with alginic acid more effective than the synthetic resin. The affinity of each of the adsorbents for sodium was not appreciably influenced by the ion concentration. The sodium adsorption in unbuffered solutions was accompanied by a marked increase in hydrogen ion concentration. Liberation of hydrogen ions by 1 g

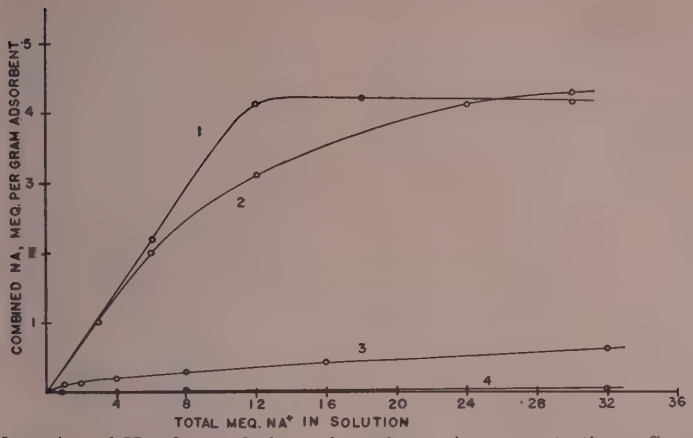

FIG. 1. Adsorption of Na^+ from solutions of varying cation concentrations. Curve 1, alginic acid and Curve 2, Natrinil, in phosphate solution pH 7.6. Curve 3, alginic acid and Curve 4, Natrinil, in unbuffered NaCl solution.

alginic acid in equilibrium with 4 meq Na^+ resulted in a lowering of pH from 6.0 to 2.2. Natrinil produced a corresponding drop in pH to 3.0.

2. *Adsorption of Na^+ from buffered solutions.* The adsorption of Na^+ from solutions containing Na_2HPO_4 and NaH_2PO_4 in a ratio to give an initial pH of 7.6 was determined as described above. Under the conditions employed conversion of alginic acid to its soluble sodium salt took place. Consequently, the extent of sodium adsorption was made by direct analysis of the dialysis bag contents. In experiments using Natrinil the bag contents were ashed and the determination of combined sodium made by analysis of an aqueous solution of the combustion residue. Adjustment of the pH of the medium to approximately that of the small intestine resulted in a marked increase in the adsorption capacity of the adsorbents (Curves 1 and 2, Fig. 1). When equilibrated with solutions containing successively higher concentrations of Na_2HPO_4 and NaH_2PO_4 having a pH of 7.6, alginic acid combined with greater amounts of Na^+ until the maximum of 4.2 meq/g was reached. The inability of this adsorbent to combine with increasing amounts of sodium when equilibrated with amounts of Na^+ above 12 meq/g indicates that the value of 4.2 meq/g is the effective sodium adsorbing capacity of alginic acid under physiological conditions. The cation combining capacity of Natrinil is approximately the same as that of alginic acid under these conditions. However, the synthetic adsorbent appears to require a greater excess of cation to bring about complete saturation.

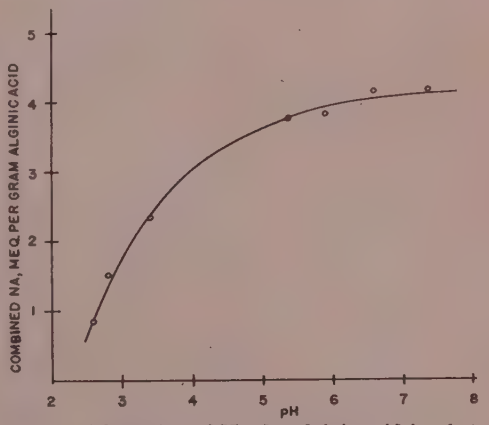

FIG. 2. Adsorption of Na^+ by alginic acid in phosphate buffer solution.

The failure of alginic acid to adsorb its capacity of sodium when equilibrated with less than 12 meq of ion is not due to a mass action type of relationship, but rather to the inability of these low concentrations of sodium phosphate to maintain the pH at a favorable level for efficient adsorption. This is apparent from the data presented in Fig. 2, which shows the sodium adsorbing capacity of alginic acid as a function of pH. In these experiments,

TABLE I. Selectivity of Alginic Acid for Sodium and Potassium at Various Cation Concentrations and Na:K Ratios.

Total cation present (meq)	Ratio $Na^+:K^+$	Combined cation (meq/g)	Ratio Na:K
12	1:1	4.1	1.1 :1
18	1:1	4.3	1.1 :1
18	5:1	4.1	4.7 :1
18	25:1	4	31.6 :1
24	1:1	4.3	1.15:1
30	1:1	4.3	1.15:1
30	5:1	4.2	4.85:1
60	1:1	4.5	1.25:1
60	5:1	4.2	5 :1
120	1:1	4	1.2 :1

alginic acid was equilibrated with 40 ml of phosphate buffer solution containing 30 meq Na^+. The phosphate mixtures were chosen to give the equilibrium pH values indicated in Fig. 2.

3. *Selectivity of alginic acid for sodium and potassium ions.* The adsorption of Na^+ and K^+ from solutions containing mixed sodium and potassium phosphates at pH 7.6 and at varying ratios of Na:K was determined. The cation-adsorbent ratios found favorable for sodium saturation at this pH were employed, that is 12 to 120 meq cation per g of adsorbent. Determinations of combined sodium and potassium were made by analysis of the dialysed bag contents as described earlier. The data in Table I indicate that the total cation adsorption from a solution containing both K^+ and Na^+ is the same as that obtained when Na^+ is present exclusively. The data also indicate that the "selectivity" of the adsorbent is determined by the cation ratio present in solution. The ratio of Na:K combined in all cases approximates that present in solution throughout the range of cation: adsorbent ratios studied.

4. *Adsorption of Ca^{++} by alginic acid.* Because of the difficulty of finding a suitable buffering agent compatible with calcium ions, calcium adsorption studies were carried out on unbuffered solutions prepared from calcium chloride and calcium hydroxide. One g of alginic acid was shaken with 200 ml of solution containing 10 meq Ca^{++}, the mixture allowed to stand overnight, and the supernatant liquid filtered and analyzed for uncombined calcium. By adjusting the ratio of calcium hydroxide and calcium chloride used it was possible to obtain equilibrium pH values from 3.0 to 11.8. Over this pH range, the calcium adsorption per g of alginic acid was 5.4 $\pm$ 0.3 meq. This value can be considered constant within the experimental error of the analytical procedure used. It is also equal to the alkali titration value of 5.2 meq/g obtained for the alginic acid employed in these studies. Natrinil was found to have a calcium adsorption capacity of the same magnitude, combining 5.5 meq/g at pH 7 and above. However, upon lowering the pH below 6, the calcium combining capacity dropped sharply, so that little if any calcium was removed from solution at pH 3 and below. In this respect the synthetic resin exchange material differs markedly from alginic acid.

Discussion. The studies reported in this paper show that *in vitro* alginic acid is capable of adsorbing sodium and potassium ions as efficiently as the synthetic cation exchange resins. The selectivity for sodium exhibited by the natural material also appears to be similar to that observed with the resins. Alginic acid, although itself insoluble in water, differs from the exchange resins in forming water soluble sodium and potassium salts. Alginic acid also differs from the resins in being broken down to a certain degree in the digestive tract(2).

The free acid form of carboxymethylcellulose was also explored for its cation adsorbing properties. In general it behaved in a similar manner as alginic acid, forming a soluble sodium and potassium salt and an insoluble calcium salt.

The use of alginic acid as a cation exchange material *in vivo* appears worthy of study. This substance, which for satisfactory sodium removal must be taken in daily doses of at least 45 g, is more palatable than the resins. The sodium alginate formed in the intestine is extremely hydrophilic and this property assists in the removal of fluid. The mildly laxative property of the sodium alginate gel also constitutes a desirable property.

The ability of alginic acid to adsorb calcium indiscriminately over a wide range of hydrogen ion concentrations may unfavorably influence cation adsorption *in vivo*. When given

orally, alginic acid could combine irreversibly with any ionic calcium present in the stomach contents. If not completely used up in this manner, it could continue to adsorb calcium on passage through the intestine. Available evidence indicates that alginic acid, in a similar manner as the resins, adsorbs cations proportional to their ionic concentration. Since considerably smaller amounts of calcium than of sodium are present in the intestine in a soluble form, a proportionally smaller uptake of calcium would be expected. For this reason, it appears probable that the danger of gradual demineralization of bone with long continued use of alginic acid would not be greater than with the resins.

Preliminary clinical trials carried out by Dr. Mulinos at the New York Medical College and by Drs. Doerner and Feldman at the U. S. Marine Hospital, Staten Island, indicate that alginic acid combines with sodium *in vivo*.

Summary. Alginic acid possesses sodium and potassium ion exchange properties which compare favorably with those of a carboxylic type cationic exchange resin. Under *in vitro* conditions alginic acid at pH 7.6 adsorbs 4.2 meq Na^+ per g. The total cation adsorption from solutions containing K^+ and Na^+ is also 4.2 meq/g and the ratio of Na:K combined approximates that present in solution. The adsorption of Ca^{++} by alginic acid is irreversible and independent of pH. The hydrophilic character of the sodium and potassium alginates may contribute to the elimination of fluid and cause laxation.

1. For a review of pertinent publications in this field, see McChesney, E. W., Dock, W. and Tainter, M. L., *Medicine,* 1951, v30, 183, and McChesney, E. W., *J. Lab. Clin. Med.,* 1951, v38, 199.

2. Nilson, H. W. and Lemon, J. M., *U. S. Dept. Int., Fish and Wildlife Service, Res. Rep.,* 1942, No. 4.

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A Nucleoprotein-Containing Fraction of Intestinal Mucosa. (19312)

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In an attempt to extract from intestinal mucosa that substance, expected to be mucoprotein, which imparts to the tissue its mucous nature, a highly viscous fraction was obtained which appears upon analysis to be approximately 50% nucleoprotein. The remainder of the fraction, not yet separated from the nucleoprotein, is primarily lipid and ash. A description of the preparative method and of the product is given here because of the simplicity of the technic and because of the biochemical demonstration (succeeding paper) of an effect of whole body X-irradiation upon this fraction of intestinal mucosa.

Preparation of the nucleoprotein fraction. The nucleoprotein fraction has been prepared from whole rat intestine and from the intestinal mucosa of rats, rabbits, and sheep. The intestines were cooled on ice as quickly as possible after removal from the animal, then were flushed with cold saline to remove fecal matter. Whole rat intestine was minced in a Latapie mincer. When rat intestinal mucosa was desired, the intact segments of intestine were slit lengthwise, then fed through a device resembling a washing machine wringer; the rollers were made of stainless steel, lightly milled to afford traction. Mucosa was obtained from the cleaned intestine of rabbits or sheep by pressing intact segments with bone spatulas. Whichever the product, whether mucosa or whole intestine mince, it was then blended in the Waring blendor with 9 volumes of N/10 NaOH. The blend was filtered through nylon (National Filter Media Co.,

* Formerly University of Chicago Toxicity Laboratory. This work was carried out under a research contract with the Atomic Energy Commission.

TABLE I. Chemical Composition of Tactoid from Intestine.

Sample		Rat (pool of several)	Rabbit (1 animal)	Sheep (1 animal)
Total N (mg/ml)	O*			1.10
	T	1.71	2.73	2.60
	S	1.61	1.76	
Total P (mg/ml)	O			.19
	T	.52	1.05	1.18
	S	.21	.26	.14
% dry wt	O			
	T	3.37	3.61	3.96
	S	2.09	1.78	1.12
Protein (mg/ml)	O			4.66
	T	1.98	9.66	5.40
	S	5.09	7.83	.89
Polysaccharide (mg/ml)	O			.15
	T		1.14	7.40
	S		.59	.25
Tyrosine (meq × 10^{-4} per ml)	O			43
	T	51.2	72.1	
	S	52.2	59.4	
Relative viscosity	O			2.74
	T	200-2000	†	
	S	1.43	1.47	

* O = original. T = tactoid. S = supernatant.
† Not done (very viscous).

No. 1002) under suction. It was brought to pH 11 with vigorous stirring and placed in the cold room (2-5°C). After a period of one to 4 days without disturbance, a separation takes place into 2 liquid layers. The upper, watery layer increases further in amount as the material remains in the cold. The lower layer is considerably more viscous and forms a cohesive mass which shrinks with the passage of time, expressing more watery fluid, and itself becoming viscous to the point of almost complete gelation. If the pH is not reduced to 11 before placing in the cold room, the two-phase separation will still occur, but it may take several weeks, at the end of which time it will be found that the pH has decreased to and below pH 11, presumably by absorption of CO_2 from the air and by reaction of the alkali with the glass vessel. The cohesive, viscous mass will hereafter be referred to as the "tactoid," a not entirely accurate usage of the expression of Freundlich(1) for the microscopic or submicroscopic particles which when properly oriented, form such a mass. We have also prepared such a tactoid from calf thymus chromosomes or whole thymus, but we have not found it possible to prepare it from alkaline solutions of rabbit kidney, lung, heart, liver or testis.

TABLE II. Phosphorus Fractions of Rat Intestine Tactoid.

Phosphorus fractions (as % of total P)	Age of tactoid, days 16	28	32
Acid-soluble P	28	24	20
Lipid P	6	3	9
Nucleic acid P	60	69	69
Phosphoprotein P	6	4	2

Chemical composition of the tactoid. The chief difficulty in characterizing the tactoid lies in its irregular composition, dependent upon time, temperature, pH, speed and time of centrifuging, if any, and undoubtedly biological factors. As the material stands in the cold room, fluid is continually expressed, and its chemical composition is thus constantly changing. Table I summarizes pertinent information on the chemical composition of alkaline blends of intestine, before and after formation of tactoid.

Phosphorus fractionations have been performed on some tactoids, by the method of Schneider(2). The results are given in Table II and provide strong evidence for the high nucleic acid content of the tactoid. In addition, a portion of sheep tactoid was extracted to remove acid-soluble and fat-soluble

materials, then treated with perchloric acid(3) to dissociate nucleic acids from protein. The soluble portion of the resulting product showed an absorption spectrum very similar to that of the nucleic acids.

The carbohydrate moiety of the nucleic acid was measured by the diphenylamine reaction(4), the orcinol reaction(5), and the carbazole reaction(6). The results indicated clearly the presence of carbohydrate of the desoxypentose type, with only a trace of pentose.

The ash content of these tactoids is quite high, usually about 25% of the dry weight. Besides the alkali metals, the only cation present in appreciable concentration is aluminum. Although it has been definitely established that the aluminum rises directly from the intestine (possibly partly from the diet) and is not a procedural contaminant, its significance in the formation of the tactoid is unknown.

Summary. A nucleoprotein-rich fraction has been obtained from alkaline blends of intestinal mucosa by placing the blend in the cold with or without adjustment of the pH to 11. After several days (or weeks, if the pH is not previously adjusted), a 2-phase liquid system becomes evident. The heavier, viscous, continually contracting phase, designated here as the tactoid, generally has a composition of approximately 50% nucleoprotein, 25% fat, and 25% ash. The nucleic acid is almost entirely of the desoxypentose type. The ash contains appreciable amounts of aluminum, the significance of which in the formation of the tactoid is unknown.

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Effect of Whole-Body X-Radiation on Rat Intestine and Intestinal Nucleoprotein (19313)

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In the preceding paper, a method was described for the isolation from intestinal mucosa of a tactoid fraction which has been identified as approximately 50% nucleoprotein. The present paper describes biochemical changes in this fraction after the animals receive a lethal dose of X-rays to the whole body.

Experimental. Since, as described in the preceding paper, the process of tactoid formation is a continuing one, for quantitative measurements it is necessary to prepare the intestine solutions, draw aliquots for analysis, etc., at some regular, though arbitrary, time. To achieve this aim, all intestines of a given experiment were quickly frozen in dry ice and acetone after cleaning. All were then thawed, blended with N/10 NaOH, filtered, and placed in the cold room as nearly simultaneously as possible. When tactoid formation appeared adequate, a control sample and its corresponding sample from X-rayed animals were centrifuged in the same centrifuge load. Animals used were Maguran male rats weighing 220-290 g. X-radiation administered was 800 r to the whole body. Controls and X-rayed animals were fasted 24 or 48 hours after the irradiation and then were sacrificed.

Results. Table I indicates that 24 hours after whole body X-radiation, the weight of intestine has decreased significantly, the rela-

* Formerly University of Chicago Toxicity Laboratory. This work was carried out under a research contract with the Atomic Energy Commission.

TABLE I. Effect of Whole Body X-Radiation on Some Properties of Rat Intestine.

	Control	X-Rayed
Wt of 3 intestines, g	22.2 ± 1.2	20.1 ± .7 (t = 2.96)
Relative viscosity of alkaline sol. of intestines	2.69 ± .17	2.02 ± .21 (t = 4.78)
Protein content of alkaline sol. of intestines (mg/ml)	5.51 ± .68	6.81 ± .98 (t = 2.13)
% of total alkaline sol. appearing as tactoid	9.3 ± 4.3	1.8 ± .7 (t = 3.36)

Data presented are mean, average deviation from the mean, and Student's t value. Each datum represents the average of six pools of three rats each.

TABLE II. Effect of Whole Body Irradiation on Components of Rat Intestine and Intestine Tactoid Fraction.

	In tactoid, 24 hr			In whole homogenate, 48 hr		
	Control	X-Rayed		Control	X-Rayed	
Dry wt	9.02 ±2.47	3.87 ±.38	(t=3.30)	22.11 ±1.14	18.03 ±1.12	(t= 3.78)
Total N				1.70 ± .06	1.21 ± .10	(t= 6.27)
Total phosphorus	.097± .027	.040±.003	(t=3.34)	.226± .007	.144± .002	(t=10.02)
Ash	.57 ± .19	.26 ±.07	(t=2.87)			
Protein	.54 ± .33	.10 ±.02	(t=2.59)			
Fat	.96 ± .36	.66 ±.18	(t=1.49)			
Polysaccharide	.134± .052	.106±.063	(t= .56)	.356± .153	.371± .085	(t= .12)

Data are expressed as g of component per 100 g fresh intestine. Each datum represents average of 4 pools of 3 rats each.

tive viscosity of alkaline solutions of intestine has greatly decreased, and the percentage of the total alkaline solution which separates as tactoid has significantly decreased. At the same time, an apparent increase in protein content is not statistically significant.

Table II presents the effect of whole body X-radiation on certain components of the tactoid prepared from animals sacrificed 24 hours after irradiation and on these same components of the whole alkaline solution prepared from the intestines of animals sacrificed 48 hours after irradiation. It will be observed that the irradiation caused significant decreases in dry weight, total nitrogen, total phosphorus, and ash, a dubiously significant decrease in protein and no significant change in fat or polysaccharide (alcohol-precipitable carbohydrate) content.

In a separate experiment, the total phosphorus of the tactoids was fractionated, and it was found that the greatest part of the loss of phosphorus was derived from the nucleic acid fraction. These data are, of course, consonant with the concept that one effect of whole body X-radiation is the degradation of nucleic acid. In the intestine, notoriously radiosensitive, this is strikingly visible chemically, as has previously been shown by Ely and Ross(1), using ultraviolet measurements.

Summary. After 800 r of whole body irradiation to rats, a nucleoprotein-rich fraction of the intestine is diminished in amount and also in its content of total phosphorus, total nitrogen, dry weight, and ash. The greatest loss of phosphorus is derived from the nucleic acid fraction. In addition, the weight of the intestine, as well as the viscosity of its alkaline solutions, is significantly decreased after whole body irradiation. These data are interpreted as substantiation of the idea that one effect of whole body irradiation is the degradation of nucleic acid.

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Adrenal Ascorbic Acid in Male and Female Rats after Total Body X-Ray Irradiation.* (19314)

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Stress situations are associated with fluctuations in the ascorbic acid content of the adrenal gland(1,2). It has been suggested that the extent of the drop in the ascorbic acid level may be a measure of the severity of the emergency situation(3). There is evidence that the drop in adrenal ascorbic acid is causally related to ACTH. We are currently interested in the effects produced by partial and total body irradiation, and in the protection, if any, afforded by the spleen, adrenal, or other organs. It was of interest, therefore, to determine the ascorbic level of the adrenals of rats after X-ray irradiation.

Methods. The animal material included 128 male and female rats (Long-Evans), 80 to 90 days of age, divided into 7 experimental and one control group. X-rays administered were generated by a 250 KV machine. The total dose was 625 r. Two filters, 0.25 Cu and 0.5 Aluminum were used. The Half-Value-Layer in copper of the filter beam, using 250 KV, was 1.05 mm. The measurements were determined in air by the position occupied by the center of the animal's body. The dose rate was 410 per min. at 60 cm. Back scatter was eliminated by the use of a cardboard box 5 cm in depth, which separated the animal from an X-ray permeable plastic table. One, 3, 6, 12, 24, 48, and 96 hours following exposure the animals were sacrificed individually by decapitation. Immediately following, the adrenals were removed, dissected free of extraneous tissue, weighed on a Roller-Smith balance, and homogenized in cold 2.5% metaphosphoric acid. The Coleman spectrophotometer was used for ascorbic acid determination(4).

Results and conclusions. The adrenals of the males showed a marked depletion of ascorbic acid after one hour. There was a rise after 12 hours with a return to normal after 48 to 96 hours. Diarrhea was observed in half of the animals in the 48-hour group and in a more severe form in all of the animals in the 96-hour group. The animals in the latter group were sluggish and exhibited priapism. Diarrhea was observed in 50% of the females after 96 hours. They were sluggish and at necropsy the intestines were observed to be distended with gas and the adrenals were a dark, cherry red color. The weights of the adrenals of the 96-hour male group were twice that of the other males. The 48- and 96-hour groups were the only ones to show loss in body weight. The average loss was 16 g in the former and 53 g in the latter (Table I). The

TABLE I. Changes in Adrenal Ascorbic Acid in Male and Female Rats (Long-Evans) Following Total Body X-ray Irradiation. Eight animals in each series.

Time, hr	Adrenal* wt, mg	Body wt, g	mg ascorbic acid/100 g adrenal with S.E.M.	P value
		Males		
U†	15.9	230	444 ± 7.3	N‡
1	17.8	228	228 ± 27.6	.0001
3	17.6	228	301 ± 22.5	.0001
6	17.4	215	396 ± 18.3	.0164
12	18.5	228	414 ± 18.3	.1336
24	17	228	493 ± 16.8	.0093
48	16.9	202	448 ± 16.8	N‡
96	30.5	187	444 ± 45	N‡
		Females		
U†	23.5	160	429 ± 6	N‡
1	23.5	156	298 ± 20.3	.0001
3	27.5	215	447 ± 6.3	.0394
6	24.7	174	483 ± 17.3	.0032
12	29.4	191	463 ± 7.5	.0004
24	34.6	218	423 ± 18.5	N‡
48	28.8	221	518 ± 7.4	.0001
96	34.9	206	430 ± 18.5	N‡

* Wt of single adrenal. † untreated. ‡ normal.

* This investigation is one phase of a study of the effects of irradiation facilitated by a Grant-In-Aid from the Atomic Energy Commission (Contract No. AT (11-1-110)). The authors take pleasure in acknowledging the technical assistance of Mr. Thomas Marinkovitch.

loss of weight was greater in the males than in the females. There was no correlation between ascorbic acid content and adrenal weight. It will be seen that although the adrenals were larger in the females than in the males, their ascorbic acid content was less (Table I). The drop in ascorbic acid was less precipitous in the female 1-hour group than in the corresponding male series and the return to the normal level was more rapid in females. Another difference between the two sexes was a secondary rise in ascorbic acid in the female. Repetition of the experiment yielded identical results. The adrenals of the male were more hypertrophic than those of the females. While there is no complete explanation of the changes which follow X-ray irradiation, the observation of Sayers and Sayers(5) that progesterone blocks ACTH release, combined with the experiments of Snyder and Wyman(6), who have shown that progesterone has twice the potency of DOCA in maintaining adrenalectomized hamsters, are of interest in that they suggest a possible explanation of the less precipitous fall in adrenal ascorbic acid in the female rat. Finally, the possibility that the gonads may play a role in this mechanism is suggested by the experiment of Wexler(7) which showed less depletion of ascorbic acid in animals with intact ovaries.

Summary. Total body X-ray irradiation is associated with a marked depletion in adrenal ascorbic acid. The decrease in ascorbic acid is more marked in the male rat than in the female.

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Tolerance of Normal and Adrenalectomized Rats for Cortisone Acetate.* (19315)

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Howard(1) has commented upon "the almost uniform finding that an organism is highly oversensitive to a hormone that it lacks, and the corollary that the normal is relatively insensitive to doses of a hormone which would seriously injure the deficient animal." Thus, the depancreatized rat has a less-than-normal tolerance to insulin(2), and removal or destruction of the thyroid decreases the tolerance for large doses of thyroxin(3). Selye(4) has reviewed the evidence that adrenalectomized animals and patients with Addison's disease are more sensitive than normal to over-dosage with 11-desoxycorticosterone acetate.

In the present experiments large doses of cortisone acetate were administered to adrenalectomized and to sham-operated force-fed rats. The adrenalectomized rats lost more weight, excreted more glucose and showed a greater incidence of gross pathologic changes than did the non-adrenalectomized rats.

Methods. Male rats of the Sprague-Dawley strain having an initial weight of approximately 300 g were used in these experiments. The animals were placed in metabolism cages and were force-fed a medium carbohydrate diet(5) by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technics and diet were modifications of those described by Reinecke, Ball, and Samuels(6). The experiments were carried

* We wish to express our appreciation to Dr. Augustus Gibson, Medical Division of Merck and Co., who supplied the cortisone acetate.

TABLE I. Comparison of Normal and Adrenalectomized Rats Given Cortisone Acetate for 21 Days.

Operation	No.	Daily dose, mg	Number showing gross pathology: Died	General infection	Lungs	Heart	Liver	Kidneys	Stomach ulcers	Avg and range*: Wt loss, g/rat	Total urine glucose/rat
Adrenx	12	5	1	0	0	0	0	11	4	37 (19-60)	6.5 (.0-20.2)
Sham	12	5	1	1	1	1	1	9	2	26 (15-41)	7.1 (.0-20.3)
Adrenx	23	10	2	5	9	3	7	18	18	59 (37-98)	51.6 (12.1-97.4)
Sham	23	10	1	3	6	2	3	13	12	47 (7-71)	30 (.0-62)

* See text for statistical calculations.

out in an air-conditioned room at a temperature of 74 to 78°F. Twenty-four hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with toluene. Urine glucose was determined by the method of Benedict(7). The rats were adrenalectomized under aseptic conditions by the method of Ingle and Griffith(8). In the sham-operated animals the adrenals were exposed but were not manipulated. The adrenalectomized rats were treated with 3 cc of beef adrenal cortex extract per rat per day during the control period only. Following a control period of 14 days, all of the rats were given twice daily subcutaneous injections of cortisone acetate (Merck) in aqueous suspension. All of the rats were subjected to necropsy either when they were found to be dying or on the 21st day following the beginning of injections.

Experiments and results. In Exp. 1, 12 pairs of adrenalectomized and sham-operated rats were given 5 mg of cortisone acetate per rat daily for 21 days. In Exp. 2 and 3, 12 and 11 pairs respectively of adrenalectomized and sham-operated rats were given 10 mg of cortisone acetate per rat daily for 21 days.

Body weight (Table I). In each of the 3 experimental groups the average loss of weight was greater in the adrenalectomized rats than in the sham-operated rats. When the data for Exp. 2 and 3 were pooled together, the average loss of weight for the adrenalectomized rats was 59.5 ± 2.7 g and the average loss of weight for sham-operated series was 47.0 ± 3.3 g. The difference of 12.5 ± 4.26 g is statistically significant.

Glycosuria. In Exp. 1 (Table I), 7 adrenalectomized rats and 8 sham-operated rats excreted glucose on one or more days during the injection of 5 mg daily of cortisone acetate. There was no significant difference in the average amount of glucose excreted by the 2 groups. In Exp. 2 (Table I) all 12 of the adrenalectomized rats and 11 of 12 sham-operated rats excreted glucose on one or more days during the injection of 10 mg of cortisone acetate daily. The average amount of glucose excreted by the adrenalectomized rats was greater than that shown by the non-adrenalectomized rats. In Exp. 3 (Table I), all 11 pairs of adrenalectomized and sham-operated rats developed glycosuria during the experiment. The average amount of glucose excreted by the adrenalectomized rats was greater than that of the non-adrenalectomized rats. The total amounts of glucose excreted by each of the rats in Exp. 2 and 3 were compared. The average for 23 adrenalectomized rats was 51.6 ± 4.2 g and the average for 23 sham-operated rats was 30.0 ± 4.1 g, a statistically significant difference of 21.6 ± 5.87 g. The distribution of gross tissue changes among the experimental groups is shown in Table I. The data for Exp. 2 and 3 are pooled together in this summary.

Survival. Deaths were related to either a rapid exacerbation of glycosuria or to a generalized infection. *Infection.* Some of the rats treated with cortisone acetate developed diffuse infections which involved several of the intrathoracic and intra-abdominal organs

as well as the pleura, the pericardium and the peritoneum. *Lung lesions.* The pathologic changes in the lungs always represented infection which varied from tiny discrete nodules to massive abscesses which caused consolidation of most of the lungs. *Heart.* Visible damage to the heart consisted of white or gray spots which penetrated the surface of the heart. *Liver.* The signs of liver damage consisted either of localized abscesses or of gray patches on the surface of the liver. In addition, the livers of rats given 10 mg of cortisone acetate daily were frequently very yellow, suggesting an abnormally high content of fat. *Kidneys.* Signs of renal pathology included a mottled appearance of the kidney with gray patches penetrating the surface. Tiny nodules representing hypertrophied tubules together with tiny pits were seen on the surface of most kidneys. In a few rats the surface of the kidney was very rough due to the presence of many pits and nodules. *Stomach.* Ulcers appeared in the pyloric portion of the stomach in many of the rats. The lesions varied from tiny ulcers and shallow erosions to a few deep bleeding ulcers which filled the lower gut with blood and caused black tarry feces.

Discussion. If the rate of secretion of steroids by the adrenal cortices were unaltered by the administration of cortisone acetate, the summation of endogenous and exogenous steroids in the non-adrenalectomized animal should produce signs of hypercorticalism more readily than does an equal amount of exogenous hormone in the adrenalectomized animal. Actually, as was first reported by Ingle and Kendall(9), the administration of exogenous cortical hormones causes a compensatory atrophy of the adrenal cortices. The present data strongly suggest that signs of cortisone acetate overdosage are more easily induced in adrenalectomized rats than in similar animals having their adrenal glands intact. What role does the atrophic adrenal gland play in determining this apparent difference in resistance to the exogenous steroid? Does it have the capacity to inactivate some exogenous cortisone or does it continue to secrete substances which either balance or inhibit the effects of cortisone?

Although moderate doses of cortisone may support a subnormal rate of growth in young adrenalectomized rats(10), the characteristic effect of large doses is to inhibit growth. It must be assumed that the normal adrenal cortex secretes one or more compounds other than cortisone or 17-hydroxycorticosterone which supports growth(11). There is some evidence that 11-desoxycorticosterone, which may or may not be secreted by the adrenal cortex in physiologically important amounts, has effects which modify those of cortisone in rats(12,4).

Signs of gross pathology following overdosage with cortisone acetate occurred somewhat more frequently in the adrenalectomized rats than in the non-adrenalectomized rats. Since there is some difficulty in appraising these changes quantitatively and objectively no claim is made that we have proven a true difference in the incidence and extent of pathology between the two groups. None of the lesions which were observed has ever been found to occur in untreated control rats.

The signs of hypercorticalism reported in these experiments are similar to those observed in rats given either an excess of ACTH (13) or of cortisone acetate(5). There are an increasing number of reports of ulcers of the gastrointestinal tract in patients given large doses of either ACTH or cortisone acetate. Ingle, Sheppard, Evans, and Kuizenga(14) first reported the experimental production of gastric ulcers by 17-hydroxycorticosterone and Ingle, Li, and Evans(15) first reported the experimental production of a gastric ulcer by ACTH.

Summary. Male rats having an initial weight of approximately 300 g were force-fed a medium carbohydrate diet by stomach tube twice daily. One rat of each pair was adrenalectomized and the other was subjected to a sham-adrenalectomy. A comparison was made of the resistance of the two groups of rats to large doses of cortisone acetate. In Exp. 1, 12 pairs of rats were each treated with 5 mg of the steroid per rat per day for 21 days. The loss of weight in the adrenalectomized animals was greater than in the non-adrenalectomized animals, but there was no group difference in the extent of glycosuria. In Exp. 2 and 3, a total of 23 pairs of rats were given 10 mg of cortisone acetate per rat

per day for 21 days. The loss of weight and the average amount of glucose excreted were significantly greater in the adrenalectomized animals. The incidence of gross pathology, especially kidney changes and stomach ulcers, was greater in the adrenalectomized animals of all 3 experimental groups. It was concluded that adrenalectomized rats are more sensitive to an excess of cortisone acetate than are non-adrenalectomized rats.

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Ovarian Retention Cysts in Hypothyroid Rats Treated with Diethylstilbestrol.* (19316)

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It was reported earlier (Janes)(1) that cystic ovaries were encountered in rats which had been thyroidectomized for 8 months and that the incidence of their occurrence was increased when diethylstilbestrol was given for 20 days at the end of the period. Recently, Bourg and Simon(2) found cystic follicles in normal rats which had been given relatively large doses of estradiol for 10 or 20 days.

The present work is a continuation of the earlier study and includes observations on more thyroidectomized rats as well as rats treated with thiourea.

Materials and methods. Two hundred and twenty-nine adult female rats of the Long-Evans strain were used. Ninety-eight of these were thyroidectomized for 8 to 12 months, and 46 were rendered hypothyroid by the administration of 0.5% thiourea in the drinking water for 8 months. The remaining 85 were normal as far as thyroid function was concerned. For the last 20 days of the experiment, some of the rats in each group were given diethylstilbestrol either as daily injections of 100 γ or were implanted subcutaneously with 10 mg pellets (95% diethylstilbestrol, 5% cholesterol). At the end of the experiment, the ovaries were fixed for microscopic study. In certain instances the pituitaries were saved and assayed for gonadotrophins in immature female Sprague-Dawley rats.

Results. Rats with a chronic hypothyroidism, resulting from the addition of thiourea to their drinking water or from thyroidectomy, had a greater number of ovarian cysts than normal rats. The incidence of cyst formation in normal rats was increased when diethyl-

* This investigation has been made with the assistance of a grant from the Central Scientific Fund, College of Medicine, State University of Iowa.

TABLE I. Ovarian Retention Cysts.

Treatment	No. rats	No. rats with cysts	% with cysts
Normal	40	1	3
Normal stilbestrol 20 days	35	3	9
" " 40 "	10	0	0
Thyroidectomized 8-10 mo	39	7	18
" 8-10 mo stilbestrol 20 days	59	32	54
Thiourea 8-10 mo	28	2	7
Thiourea 8-10 mo stilbestrol 20 days	18	6	33

stilbestrol was given for 20 days, but the cysts became even more numerous when this estrogen was given to the hypothyroid animals (Table I).

Grossly the cysts appeared as clear vesicles and varied in size (Photomicrographs were shown in earlier paper, Janes(1). The cysts were classified as such only if they were at least twice the size of mature follicles. In many cases the cysts measured as much as 8 mm in diameter. The granulosa cells originally present around the follicles disappeared during cyst formation and the cysts were lined with simple squamous epithelium. The theca interna adjacent to the cysts was luteinized in rats which had received diethylstilbestrol but such luteinization was generally lacking in cystic ovaries of untreated animals. In some rats given diethylstilbestrol pathologic changes such as hydrosalpinx, hydrometra or pyometra were present along with cystic ovaries. This was particularly prone to occur in the hypothyroid rats.

In order to observe the condition of the ovaries during the estrogen injection phase, some animals were laparotomized on the 10th day. At this time the ovaries showed cysts which were not as large or as numerous as at the end of the 20-day period of estrogen treatment.

An effort was made to determine what effect a more prolonged treatment with diethylstilbestrol might have on the structure of the ovaries. One hundred gamma of diethylstilbestrol was given daily to 10 normal rats for a period of 40 days. The ovaries were larger than normal in these rats but contained only a few healthy corpora lutea and the medium and large follicles showed atretic changes. However, no ovarian cysts were found (Table I). This indicates that the hypothyroidism is prerequisite for the cystic changes which are accentuated by the estrogen treatment.

The chronic hypothyroidism lead in many instances to atrophic ovaries. Other ovaries contained only a few small follicles and an occasional corpus luteum. Histologically there was no evidence of granulosa cell or other follicular stimulation. Assays of the pituitaries from rats with cystic follicles failed to reveal any significant differences in gonadotrophic hormone content so there is no evidence that changes in gonadotrophic hormones might account for the cyst formation.

Although the conditions of the experiments were not identical, Bourg and Simon(2) found some evidence of retrogressive changes in the ovaries of normal rats which had received large doses of estradiol. It appears, therefore, that the cystic ovaries which have been observed, may not result from pituitary stimulation, but are degenerative changes concomitant with chronic hypothyroidism and estrogen therapy.

Summary. Ovarian cysts were encountered in rats with a chronic hypothyroidism but their frequency was increased when such animals were given diethylstilbestrol. The cyst formation is probably associated with degenerative changes in the ovary.

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A Simplified 60 Cycle Carrier System Capable of Measuring Low Pressures.* (19317)

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Amplification systems for the recording of pulsatile pressure changes using a transducer device have had disadvantages in our hands in ease of operation, stability of the recording mechanism and lack of sensitivity. Previous investigators(1-3) have demonstrated the fidelity and accuracy of resistance wire manometers in the registration of pressure changes derived from intracardiac catheterization technic. Recently, West and associates(4) have described an "inexpensive" carrier amplifier for use with a resistance wire-type manometer and indicated the advantages of such an A.C. amplification system. The following section describes a greatly simplified 60-cycle carrier system of markedly increased sensitivity, which can be phase sensitive and has given satisfactory biological records using a direct recording device without optical magnification.

Description of system. The components of our recording equipment are a Statham P23 A† pressure transducer, a 60-cycle carrier supply system, the balancing network, amplifier, power supply and recorder. Circuit diagrams (Fig. 1 and 2) present the arrangement and design of the apparatus. The power supply is the conventional 400 volt nonregulated and 250 volt regulated type. Line frequency of 60 cycles is fed into the transducer by means of a filament transformer. The transducer output is then fed into a 500 ohm line to grid transformer coupling to a high gain,

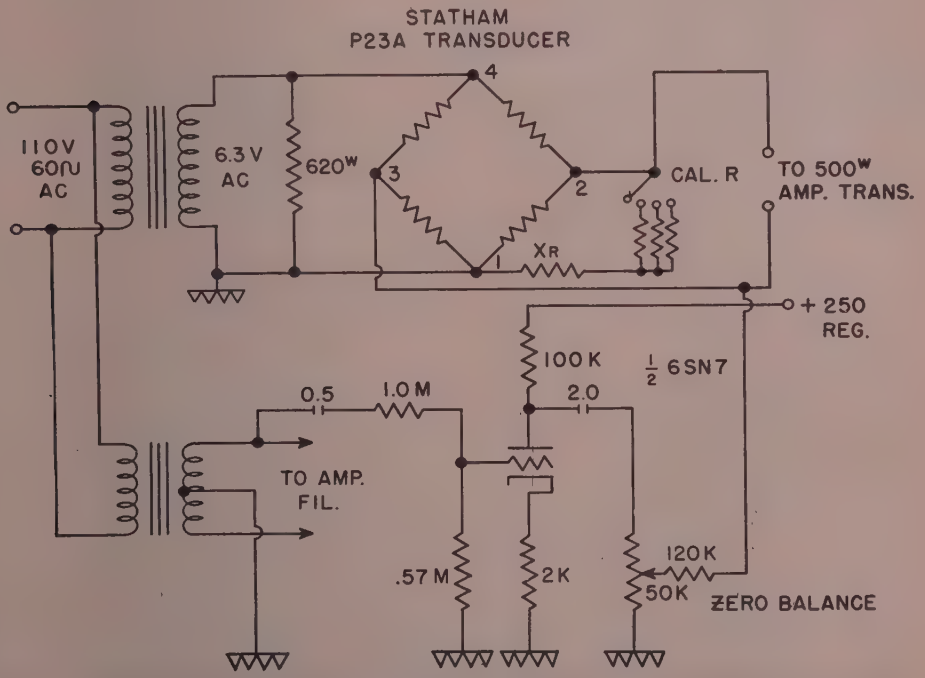

FIG. 1. Circuit showing 60 cycle carrier system and P23 A Statham Transducer with phasing and balancing network.

* Supported in part by a grant from the Damon Runyon Memorial Fund.

† Statham Laboratories, Inc., 9328 Santa Monica Blvd., Beverly Hills, Calif.

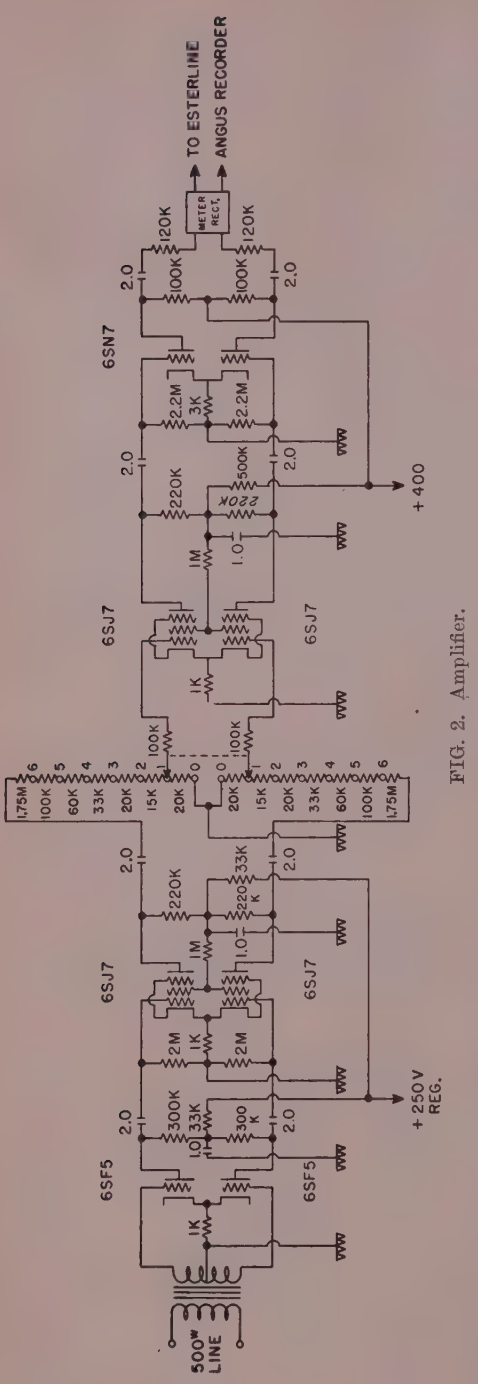

FIG. 2. Amplifier.

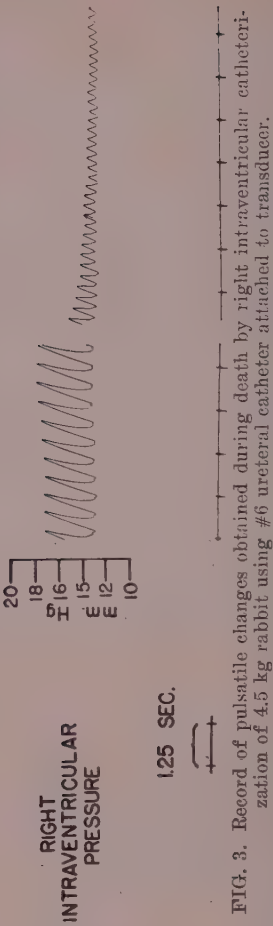

FIG. 3. Record of pulsatile changes obtained during death by right intraventricular catheterization of 4.5 kg rabbit using #6 ureteral catheter attached to transducer.

push-pull amplifier. A zero balancing system is also fed into this coupling transformer which acts to balance out the output of the transducer when no pressure is being applied. The signal is picked off the filament transformer used to operate the amplifier filaments. Proper phase is determined by interchanging the primary leads. The zero balancing control is a potentiometer on the output of the balancing amplifier tube. By unbalancing this control or zero set in the proper direction either positive or negative pressures may be recorded; this makes the system phase sensitive.

Calibrating resistors may be selected to give adequate calibrating points when the gain switch of the amplifier is turned through the various ranges. The amplifier used in this system gave the following full scale linear readings: Scale 1, 100 mm Hg; Scale 2, 60 mm Hg; Scale 3, 40 mm Hg; Scale 4, 20 mm Hg;

Scale 5, 10 mm Hg; and Scale 6, 6 mm Hg. The output of this amplifier is then fed into a meter type copper oxide rectifier coupled to a one milliampere direct current Esterline Angus recorder which is capable of giving a direct graphic record.

Remarks. This carrier system is markedly improved over those previously described in that it has a simplified A.C. source for the transducer, a phase sensitive balancing network and greater sensitivity. Operation is uncomplicated by instability and readings as low as 0.05 mm Hg can be recorded.

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Free Amino Acids in the Developmental Stages of the Mosquito.* (19318)

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The relatively few studies on the amino acids of insects have largely dealt with a single developmental stage, usually immature, and the hemolymph has been the principal body fluid subjected to analysis. Consequently, very little is known concerning the changes in amino acid levels that occur during proteolysis throughout the life cycle of an insect. Since the mosquito has a relatively short life span and exhibits very rapid growth, it was felt that this insect would afford both appropriate and interesting material for the study of the variation in free amino acids occurring during metamorphosis.

Materials and methods. The mosquito species employed were *Culex quinquefasciatus* and *Aedes aegypti* which were reared in the laboratory. Egg rafts, 4th-stage larvae, pupae, adult males and females were used. The larvae were fed a standard diet consisting entirely of finely powdered dog chow. The larvae and pupae were washed with several changes of distilled water and the excess removed with blotting paper prior to weighing. Unfed adult male and female mosquitoes were collected 2 to 3 days after emergence and lightly anesthetized with chloroform. Approximately 200 mg of each stage was weighed and homogenized in a tissue grinder with 1 ml of water. The material was centrifuged and the supernatant retained. Protein precipitation was carried out by adding one volume of 95% ethanol to about 5 ml of sample and centrifuging. The supernatant was treated with 3 volumes of chloroform (15 ml) with subsequent centrifugation at a low speed, and the aqueous fraction was removed for amino acid determinations.

The paper chromatographic method(1) was employed for determinations of free amino acids in the mosquito extracts. Whatman filter paper No. 4 was used with water-saturated phenol as the first solvent and 2,4-lutidine (3 parts to 1 part water) as the second. Standard amounts of extracts were delivered to the filter paper for the one-dimensional chromatograms and represented 12.5 mg or approximately 6 mosquitoes, while 40 mg was used for the 2-dimensional runs. The amino acids were revealed by spraying the filter paper with a solution of 0.2% ninhydrin in water-saturated butanol and heating it for approximately 15 minutes at 80°C.

Results. Free amino acids found in the eggs, larvae, pupae and adult mosquitoes are

* This investigation was supported (in part) by a Research Grant from the National Institutes of Health, Public Health Service.

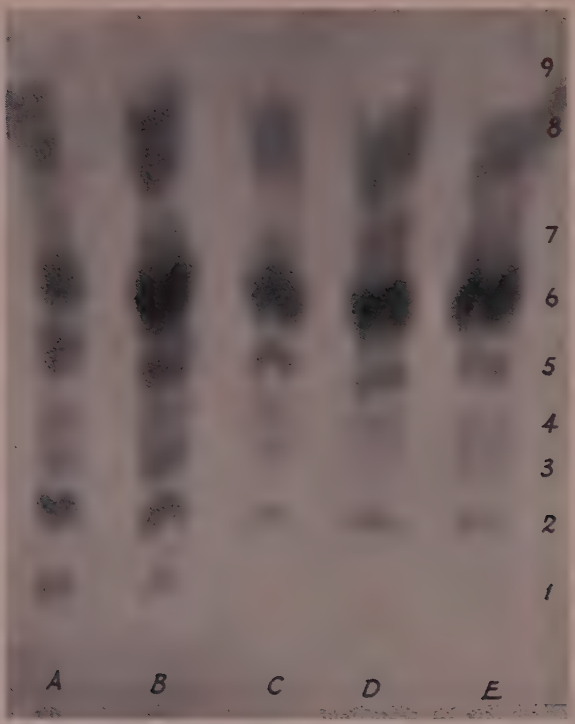

FIG. 1. Chromatogram of extracts of *C. quinquefasciatus*. A, eggs; B, larvae; C, pupae; D, adult females; E, adult males. (1, aspartic acid; 2, glutamic acid; 3, taurine; 4, serine and glycine; 5, threonine and lysine; 6, tyrosine, alanine and B-alanine; 7, valine and arginine; 8, tryptophan, leucines, methionine and histidine; 9, proline).

as follows: alanine, glutamic acid, β-alanine, isoleucine, leucine, taurine, proline, histidine, serine, valine, methionine, threonine, lysine, arginine, aspartic acid, glycine, tyrosine, and tryptophan. The relative concentrations of amino acids in the various stages of *C. quinquefasciatus* are indicated in Fig. 1 by the different intensities of the spots. The larvae appear to have the highest concentration of amino acids followed closely by the adult mosquitoes. The amino acid concentration in the extract of pupae is the lowest of all; however, 2-dimensional chromatograms revealed that this stage has considerably more alanine and tyrosine than any of the others. The eggs contained relatively small quantities of alanine and large amounts of aspartic and glutamic acids. Furthermore, aspartic acid appeared in chromatograms of the eggs and larvae, but not in those of the pupae and adults at the level of concentration used. Likewise, glutamic acid is present in considerably larger amounts in the extracts of the eggs and larvae. A comparison of the 4th-stage larvae, pupae and adults of *A. aegypti* (Fig. 2) in which the pupae were only a few hours short of maturity shows that there is a marked decrease in the intensity of all the amino acid spots in extracts of the latter with the exception of glutamic acid which occurs in rather large quantities. The adult male and female mosquitoes of both species presented essentially the same chromatographic picture.

Discussion. The level of free amino acids in the various developmental stages of the mosquito seems to be rather variable depending in part on the proximity to molting. In preparation for this process, it may be that amino acids of the pupae, at least, are utilized more rapidly than they can be replaced, perhaps due to a lowered metabolic rate in this non-feeding stage. Shortly after metamorphosis, however, the free amino acids seem to be restored rather rapidly to their original

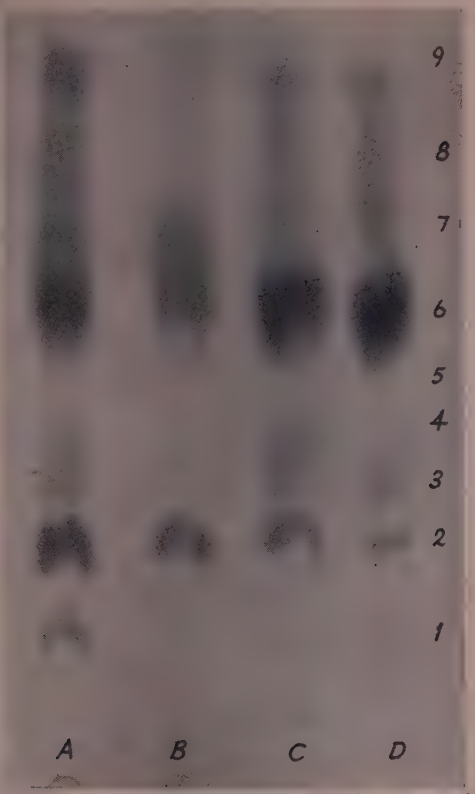

FIG. 2. Chromatograms of extracts of *A. aegypti*. A, larvae; B, pupae; C, adult females; D, adult males. (1-9 as above).

level. It is interesting to note that tyrosine is present in larger amounts in the pupae inasmuch as this amino acid is known to play an important role in the darkening or tanning of the insect integument.

The present observations are partly in accord with those of Agrell(2) who has found that although the amino acid concentration in *Calliphora erythrocephala* is comparatively constant throughout pupal development, an appreciable drop occurs at 60 to 80% of the pupal time. Maluf(3), however, states that the amino acids of the blood of pupae are highest and that this is probably due to proteolysis occurring during histolysis. The significance of his conclusions, however, is open to question since they are apparently based upon determinations of only three species of larvae and four species of pupae belonging to a single order.

Golberg and De Meillon(4) have established that the amino acids essential for mosquito larvae (*A. aegypti*) are glycine, leucine, isoleucine, histidine, arginine, lysine, tryptophan, threonine, phenylalanine and methionine. The only one of these which was not detected in the larvae used in the present study is phenylalanine, however, these authors note that the larvae seem equally capable of utilizing either phenylalanine or tyrosine. Phenylalanine was also not found in the body fluids of adult mosquitoes(1). Although tryptophan was not revealed by paper chromatographic methods in either the larvae or the pupae, its presence was ascertained by the method of Shaw and McFarlane(5).

Summary. (1) The free amino acids of mosquito eggs, larvae (4th-stage), pupae and adults (males and females) were extracted with ethanol and identified by paper chromatography. The following amino acids were found in the various stages studied: alanine, glutamic acid, β-alanine, isoleucine, leucine, taurine, proline, histidine, serine, valine, methionine, threonine, lysine, arginine, aspartic acid, glycine, tyrosine, and tryptophan. (2) Extracts of pupae contained lower concentrations of most amino acids than the other stages, with the greatest drop occurring shortly before emergence of the adults. Other quantitative variations from one stage to another involved only 2 or 3 amino acids.

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Production of Positive Antiglobulin Serum Test in Rabbits by Intraperitoneal Injection of Homologous Blood.* (19319)

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Studies of acquired hemolytic anemia indicate that the red cell sensitizing agent is a fraction of plasma protein similar in many ways to isoantibodies, but it is not known what conditions are necessary for its production. Since it appears to be an antibody specific for human red cells, attempts to produce the disease experimentally should include attempts to sensitize the animal to an antigen in autologous red cells. Attempts to produce autoantibodies for red cells are not new. Troisier(1) reported that in hemothorax and hemoperitoneum autohemolysins appeared in the blood and gradually increase in titer. Ludke(2) produced iso- and auto-lysins in 9 of 11 dogs by reinjecting lysed red blood cells which were obtained from the same dog. Recently Wagley and Castle(3) showed agglutination in anti-dog rabbit serum of the red cells of one of 4 dogs which had been injected with a modified Freund antigen red cell mixture. The application of the antiglobulin serum technic(4) in the experimental approach to acquired hemolytic anemia is an advance over former methods since it is the most reliable technic yet devised for detecting sensitization of red cells. Most antibodies found in acquired hemolytic anemia do not produce hemolysis with complement fixation *in vitro* and are usually weak agglutinins even in colloidal media and can be demonstrated only with antiglobulin serum. It may be that antibodies produced in animals by experimental methods will exhibit the same properties.

During a study of a patient with anemia secondary to intraperitoneal bleeding from a ruptured ectopic pregnancy, a transient positive antiglobulin test was observed. In an effort to simulate this occurrence in animal experiments, rabbits were given intraperitoneal injection of their own blood after removal by cardiac puncture. The red cells of the rabbits so injected were tested for sensitization in anti-rabbit guinea pig serum. Other rabbits received injections of rabbit red cell stroma that had been incubated with filtrate from hemolytic streptococcus cultures or with emulsions of rabbit liver or kidney.

Preparation of anti-rabbit globulin guinea pig serum (ARGGS). Five-tenths cc of pooled rabbit serum was injected subcutaneously once a week for 4 succeeding weeks into 4 guinea pigs. A one-week resting period was followed by 4 succeeding weekly injections. Blood was obtained by cardiac puncture or the animal was sacrificed and bled 10 days after the final injection. The serum was separated from the clot and heated to 56°C for 30 minutes and absorption of red cell antibodies was carried out by successive incubation at room temperature with half the volume of pooled red cells from 8 normal rabbits. After two absorptions the serum no longer agglutinated red cells from normal rabbits. The activity of the absorbed anti-rabbit serum was demonstrated by preciptin tests with normal rabbit serum. A positive precipitation reaction was obtained up to the 1-100 dilution of the rabbit serum with a 1-5 dilution of guinea pig serum. Control guinea pig sera gave negative results. Preliminary to injection of the test rabbits their cells were tested and found to be negative to ARGGS.

Intraperitoneal injection of homologous blood in 12 rabbits. A total of 12 rabbits were used. The usual aseptic technics were employed to prevent contamination, and none of the rabbits showed signs of infection. The first group consisted of 4 rabbits, each of which received intraperitoneally 20 cc of whole blood, which was obtained by cardiac puncture once a week for 4 weeks. The blood was injected immediately after withdrawal from the heart without anticoagulant. Once or twice a week during the period of injection

* This work was supported by a grant from the U. S. Public Health Service.

ne animal's RBC were tested with ARGGS, nd the 28th day after the first injection, the ntiglobulin test was positive to a 1/20 dilu- ion in 2 of the rabbits. Injections of red ells were stopped and the positive reaction ersisted 14 days. The *second group* con- sted of 8 rabbits which received weekly injec- ions for 3 weeks, then biweekly injections for weeks. At the end of the 3rd and 4th week he washed RBC of one rabbit were agglutin- ted in a 1/5 and 1/10 dilution of antiglobu- n serum. No other evidence of immunologi- al abnormality such as the development of uto- or isohemolysins, or agglutinins in nor- nal or acidified serum was demonstrated n the sera of rabbits whose red cells showed positive antiglobulin serum test. There was o evidence of anemia, reticulocytosis or of ncrease in osmotic or mechanical fragility.

Injection of red cell stroma plus bacterial ltrate, kidney emulsion or liver emulsion. Red cell protein was collected as stroma in he Sharples centrifuge after hemolysis of vashed rabbit red cells in 10 volumes of dis- illed water. One volume of undiluted B- emolytic streptococcus filtrate plus 1½ vol- mes of 5% red cell stroma were incubated t 37°C for 2 hours, then stored at –30°C. Of 14 rabbits 4 received 2.5 cc red cell stroma treptococcus filtrate mixture, 2 received 1.5 c 5% red cell stroma, and 2 received 1 cc treptococcus filtrate by daily subcutaneous njection for 30 days. Four rabbits received .5 cc 5% red cell stroma plus 2 cc rabbit idney emulsion and 2 rabbits received the ame amount of red cell stroma plus 2 cc f rabbit liver emulsion twice a week for 4 veeks by subcutaneous injection. Hemoglo- in, reticulocyte count, and antiglobulin tests vere done before, during and after the injec- ions for a 3 month period. However, there vas no significant change in hemoglobin and eticulocyte count and the red cells continued o show a negative antiglobulin serum test.

Discussion. There is only slender evidence or the postulate that the red cell antibody f acquired hemolytic anemia is a specific esponse to an antigen in the red cell. Per- haps the best evidence that a foreign substance may form an antigenic complex which results in an antibody capable of destroying the tissue part of the complex are the observations of Ackroyd(5) in sedormid thrombocytopenic purpura. However, the antibody in this case is not active for the thrombocyte alone. It may be that a foreign substance also plays a role in the initiation of acquired hemolytic anemia. It is a clinical impression that there is a high incidence of infection, trauma and medication in the background of patients with the disease.

The observation that intraperitoneal injection of red cells may result in a positive antiglobulin serum test suggests that relatively bland treatment of red cells may result in modification of surface structure sufficient to provoke antibody production. The nature of possible changes in red cell structure produced by intraperitoneal injection is not known, but it is conceivable that tissue enzymes or free fatty acids in lymph may produce significant alterations.

Summary. (1) Three of 12 rabbits receiving intraperitoneal injections of blood immediately after removal from the heart developed a transiently positive antiglobulin serum test (Coombs test) with a reagent made by injecting rabbit serum into guinea pigs. None of the 3 developed evidence of a hemolytic anemia. (2) Rabbits receiving injections of rabbit red cell stroma which had been incubated with bacterial filtrate or emulsions of rabbit liver or kidney failed to develop positive agglutination tests with the same reagent.

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Hepatic Necrosis Due to Bromobenzene and its Dependence upon Available Sulfur Amino Acids.* (19320)

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In many instances of hepatic injury, it is difficult to decide whether an excess of a toxic factor or a deficiency of an essential factor is the underlying cause. This problem, originally raised by the nutritional hepatic injury(1) and the demonstration of the importance of sulfur amino acids(2), has been recently stressed by the effect of ethionine upon the liver. This substance, being an analogue of methionine and thereby a possible inhibitor of protein synthesis(3), produces a fatty liver in female rats(4,5). In an attempt to find other examples of hepatic injury produced by depletion of amino acids, the acute effect of bromobenzene upon the liver was studied. The rationale for this experiment was the coupling of bromobenzene with cysteine to form bromobenzylcysteine, which is acetylated and excreted as mercapturic acid in the urine (6,7). Since methionine may serve as source for cysteine, bromobenzene could deplete the body of both sulfur amino acids. Therefore, the influence of preceding protein depletion and of sulfur amino acid supplements upon the hepatic injury was also investigated.

Material and methods. Wistar strain white rats were divided into 8 groups. All of them, except one control group, received by intraperitoneal injection 0.05 ml of bromobenzene per 100 g body weight, dissolved in 0.20 ml of corn oil. The fasting time before bromobenzene administration, the protein content of the diet fed for two weeks before the fasting period and the sex of the animals, varied (Table I). Supplements of methionine or cysteine were given in 4 doses 12 hours apart during the fasting and experimental period (*i.e.*, 16 hours before to 32 hours after the bromobenzene administration). All animals were killed 48 hours after the administration. The degree of hepatic necrosis in the histologic specimen was graded from 1+ to 4+ taking the estimated percentage of necrotic area of the lobule as criterion.

Results. In unprotected rats the centrolobular zone revealed necrosis 48 hours after the intraperitoneal injection of bromobenzene. This was characterized in some instances by diffuse coagulation and eosinophilia of the cytoplasm of the liver cells, the nuclei of which

TABLE I. Survival and Degree of Hepatic Damage 48 Hours After Intraperitoneal Injection of Bromobenzene to Rats.

No. of rats							
Surviving	Dead	Sex	Protein content of diet preceding fasting, %	Fasting preceding bromobenzene admin., hr	Bromobenzene inj. intraper., ml/100 g body wt	Supplement inj. intraper., 300 mg per 100 g body wt	Mean hepatic damage in surviving rats in +
24	2	♀	16	16	.05	—	3.2
13	1	♀	16	16	.05	Methionine	.9
9	0	♀	16	16	.05	Cysteine	1
7	0	♀	16	0	.05	—	1.9
10	4	♀	8	16	.05	—	3.7
7	8	♀	16	88	.05	—	3.9
11	1	♂	16	16	.05	—	3.3
5	0	♀	8	88	—	—	0

* Supported by a grant from the Dr. Jerome D. Solomon Memorial Research Foundation.

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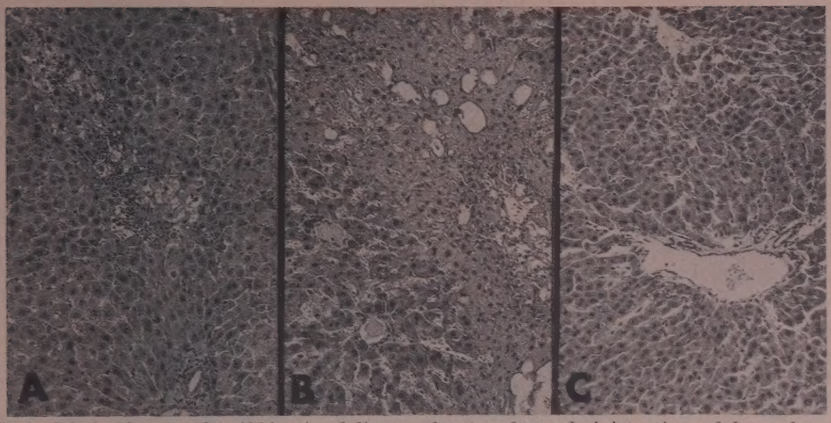

FIG. 1. Photomicrographs (220 ×) of livers of rats after administration of bromobenzene. A. Necrosis and disappearance of liver cells in the center of the lobule, which is infiltrated by inflammatory cells. The liver cells surrounding the necrosis reveal hydropic swelling. B. Disappearance and necrosis of the liver cells in the greater part of the lobule with only a small rim of liver cells remaining in the periphery. Extensive hemorrhage and dilatation of some sinusoids. C. With simultaneous administration of methionine. Intact structure.

were pyknotic. In other places the central zone showed complete disappearance of the liver cells and collapse of the framework which was surrounded by red cells intermingled with a few partly phagocytosing white cells. Some of the neighboring cells were hydropic. The Kupffer cells were proliferated and revealed phagocytosed material including non-iron-containing and non-fluorescent pigment. In the vicinity the architecture of the liver cell plates was distorted but little attempt at regeneration was noted (Fig. 1 A). In severe cases only a small rim of normal liver cells was preserved in the periphery of the lobule and the necrotic areas showed extensive hemorrhages (Fig. 1 B).

These changes were almost completely suppressed by supplementation of the diet with methionine (Fig. 1 C), and cysteine. Some protection was given by feeding the animals throughout the experiment. The severity of the liver damage was significantly enhanced and the mortality increased by either lowering the protein content of the preceding diet or increasing the fasting period. There was no difference in susceptibility to bromobenzene between males and females. The control group, kept on the low-protein diet and then fasted for 88 hours without the administration of bromobenzene, did not show significant liver damage or any mortality.

Comments. The extent of the acute centrolobular necrosis, which in severe instances is associated with hemorrhagic destruction of the lobular parenchyma, depends apparently upon the available protein stores, since the same dose produces far more extensive lesions if starvation or a low-protein intake precedes the administration of bromobenzene. The amount of cysteine available for coupling with the aromatic substance appears to be the determining factor. This is supported by almost complete prevention of the lesion by preceding administration of either methionine or cysteine in high doses. It remains to be proven that the mercapturic acid excretion is increased following the acute bromobenzene administration and that it rises even more if the hepatic lesion is prevented by the administration of either sulfur amino acid. In chronic experiments it has been proved that sulfur amino acid administration prevents on one side the growth inhibition produced by bromobenzene feeding(8,9) and increases, on the other side, urinary mercapturic acid excretion(10). The fact that the acute hepatic necrosis produced by bromobenzene injection can be prevented by the administration of sulfur amino acids, permits two interpretations: The lesion could be produced by acute deficiency of sulfur amino acids which are drained from the body during the formation

of mercapturic acid, detoxification taking preference over physiologic requirements. The massive hepatic necrosis produced by yeast diets and prevented by sulfur amino acids simulates morphologically the picture produced by bromobenzene. On the other hand, a primary hepatotoxic effect of bromobenzene cannot be excluded in view of the hepatic necroses produced by other halogenated hydrocarbons like carbon tetrachloride and chloroform, even though fatty changes present in those are missing in the bromobenzene lesion. In this case, the reported findings could be explained by a dependence of the extent of the necroses on the amount of bromobenzene not detoxified because of relative lack of sulfur amino acids. In both instances the bromobenzene lesion is a combination of a toxic and deficiency effect. How far similar mechanisms can be invoked to explain the toxicity and detoxification of other substances which produce acute liver damage, has to be investigated.

Summary. Intraperitoneal administration of bromobenzene produces acute centrolobular hepatic necrosis, its severity depending on the preceding protein depletion. Methionine and cysteine given prior to bromobenzene administration prevent the lesion. The lesion is an example of interlocked toxicity and deficiency effect, since bromobenzene drains sulfur amino acid from the body.

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